Heart fatty acid-binding proteins : role in cardiac fatty acid uptake and marker for cellular damage

HEART FATTY ACID-BINDING PROTEINS

Role in cardiac fatty acid uptake and
marker for cellular damage

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Nieuwenhoven, Frans A. van

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PROEFSCHRIFT

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Franciscus Arnoldus van Nieuwenhoven

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Promotor :

Prof.dr. G.J. van der Vusse

Co-promotor :

Dr. J.F.C. Glatz

Beoordelingscommissie :

Prof.dr. F.C.S. Ramaekers (voorzitter)
Dr. W.Th. Hermens
Prof.dr. D.J. van der Horst (Universiteit Utrecht)
Prof.dr. F. Spener (Universiteit Münster, Duitsland)
Prof.dr. R.F.A. Zwaal

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Dit proefschrift wordt opgedragen
aan mijn ouders en Elianne

Ter herinnering aan Gert-Jan

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The heart is composed of several cell-types each of which has its own specific function. About three quarters of the heart mass is made up of muscle cells, the cardiomyocytes, which have the contractile properties that enable the heart to perform its pumping activity. The other 25% of cardiac volume is taken up by the lumen and the cells from blood vessels, mainly consisting of endothelial cells and smooth muscle cells, and interstitial space containing a.o. fibroblasts, macrophages and the extracellular matrix (2,16).

The energy required by the heart for muscular contraction is supplied by the metabolic conversion of a variety of substrates including long-chain fatty acids (FA), glucose and lactate (5,28). Under physiological conditions the main energy source of the cardiac muscle cell (cardiomyocyte) is FA. Since de novo synthesis of FA is low in heart tissue, the bulk of FA are derived from the circulation (28). Fig 1.1 shows the route of FA from blood plasma to the mitochondrial matrix of the cardiomyocyte, in which compartment oxidation of FA occurs.

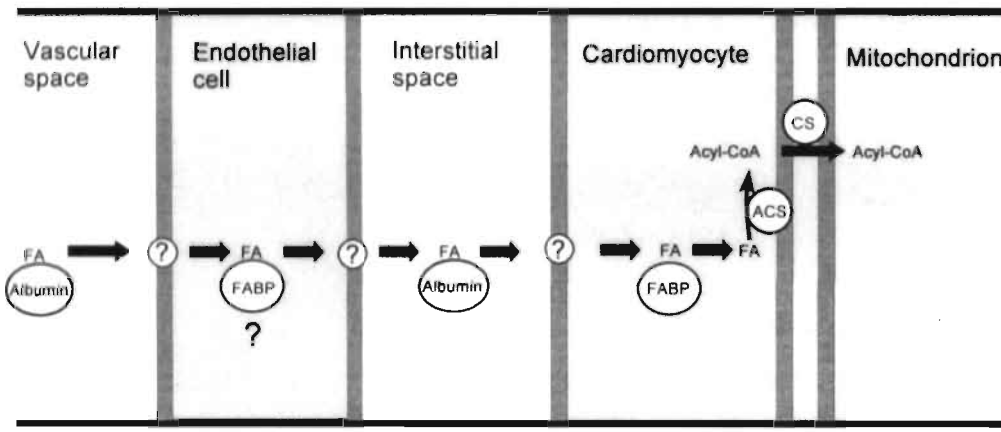

Figure 1.1 : *Highly schematic presentation of the route of transport of long chain fatty acids (FA) from the circulation through several biological compartments to the site of oxidation in the mitochondria of cardiomyocytes. FA, long chain fatty acids; FABP, heart-type fatty acid-binding protein; ACS, acyl-CoA synthetase; CS, carnitine shuttle.*

Because of their long hydrophobic tail, FA are poorly soluble in aqueous solutions. However, special binding proteins are present to enhance FA solubility. In blood plasma, FA are bound to albumin or are incorporated into triacylglycerols which in turn are part of the lipoprotein particles. FA can be taken up by the heart directly from albumin or they can be released from the triacylglycerols by the enzyme lipoprotein lipase which is present at the luminal membrane of endothelial cells (28).

The first barrier of FA on their route from the vascular space to the cardiomyocytes is the endothelial cell. In heart, the intercellular clefts between endothelial cells are only about six nanometer in width (12) and transport of albumin-FA complexes through the clefts is too slow to explain the observed FA uptake rates (4). FA have to cross the luminal membrane, the cytoplasm, and the abluminal membrane of the endothelial cells to reach the interstitial space. The interstitial fluid contains albumin and it is likely that in this compartment FA are mainly transported via diffusion of albumin-FA complexes. The next constraint in the FA-transport is the plasma membrane of the cardiomyocytes

(sarcolemma). In literature it is a matter of debate whether transport of FA across the sarcolemma and also across the endothelial membranes and the plasma membranes of other cell-types occurs via diffusion across the lipid bilayer or via protein-mediated processes (6,7,24,25).

After FA have crossed the sarcolemma, they have to reach the mitochondrial outer membrane for further metabolisation. In the cytoplasm of cardiomyocytes a small, 15 kDa protein is present with a high affinity for FA and therefore called heart-type fatty acid-binding protein (H-FABP) (11). This H-FABP is part of a family of low molecular weight lipid-binding proteins and is most likely involved in the transport of FA from the sarcolemma to the mitochondria (3,21). At the outer membrane of the mitochondria, FA are converted into acyl-CoA by the enzyme acyl-CoA synthetase. Subsequently the acyl part can be transported into the mitochondria through the carnitine shuttle and stepwise degradation then occurs via the ß-oxidation and the citric acid cycle. Alternatively, the acyl moiety of acyl-CoA can be incorporated into triacylglycerols or phospholipids (28).

When part of the heart is deprived of blood flow (ischemia), the cells in the affected tissue receive less oxygen and metabolic substrates. At the same time waste products accumulate because they are not carried away. When ischemia sustains, the cells will be irreversibly damaged and the plasma-membrane will rupture. Intracellular proteins then leak out of the cell into the interstitial compartment and eventually also appear into the vascular space. Hence, detection of these cardiac proteins in blood plasma can be used to assess myocardial injury. H-FABP is one of the proteins used to detect the occurrence and to estimate the extent of myocardial injury.

OUTLINE OF THE THESIS

The present thesis consists of two parts. The first part (chapters 2 through 5) deals with the issue of FA uptake in the heart and the potential role of FA-binding proteins in this process. The second part (chapters 6 to 8) describes studies on the release of intracellular proteins upon myocardial cell damage.

A short introduction into the FA-metabolism of the heart is presented in **chapter 2**, with emphasis on the existence and significance of membrane-associated and cytoplasmic fatty acid-binding proteins.

As mentioned earlier, endothelium forms the first barrier in the uptake of FA by the heart. It was hypothesized that endothelial cells contain a FABP which would facilitate the transport of FA through the endothelial cytoplasm (4,8). Several groups investigated this hypothesis, and there was substantial debate about the presence and the concentration of H-FABP in endothelial cells from heart tissue (19,22,23). Furthermore it was not clear whether other types of FABP are present in cardiac endothelial cells. Therefore, we studied the existence of FA-binding activity in the endothelial cell cytoplasm with a newly developed method. In addition, molecular biological and immunochemical assays were used to measure the concentrations of two FABP-types, H-FABP and liver-type (L-) FABP, both of which are known to be present in more than one type of tissue (9). The results of these studies and consequences for the hypothesis of FABP-facilitated FA-transport through the endothelium are described in **chapter 3**.

Chapters 4 and 5 handle the problem of transsarcolemmal transport of FA and the suggested role of a specific membrane-bound protein called fatty acid translocase (FAT) in this process. FAT was hypothesized to be involved in FA-uptake into adipocytes

(13,14), and was also found to be present in myocardial tissue in 1993 (1). **Chapter 4** describes a study on the co-expression of FAT and H-FABP in rat heart and skeletal muscles. We reasoned that proteins involved in the same process of FA-uptake would show the same tissue distribution and regulation of expression. To find a more definite proof for the role of FAT in myocardial FA-uptake, transfection of FAT into cell-line H9c2, derived from embryonic rat heart tissue (17), was performed. H9c2-cells normally do not express FAT and the working hypothesis was that expression of FAT by stable transfection would increase the FA-uptake into these cells. Results of this study are described in **chapter 5**.

The second part of this thesis deals with the use of intracellular proteins, especially H-FABP, as markers for cell damage. During cell damaging conditions, proteins leak out of the cells, and detection of the released proteins can be used as indicator for loss of cellular integrity. **Chapter 6** gives an overview on the current knowledge of protein leakage from damaged cells or tissues. Because conflicting opinions exist in literature whether small proteins are released earlier from damaged cells than are large proteins (20,26), we studied the release of a selected number of proteins, ranging in molecular mass from 15 to 140 kDa, from isolated neonatal cardiomyocytes under two different cell-damaging conditions. Results of this study are presented in **chapter 7**.

Assessment of H-FABP in blood plasma can be used to determine the occurrence and approximate the extent of myocardial tissue injury in humans after a myocardial infarction (10,18,27). One of the most important characteristics a protein should have to be a biochemical marker for heart muscle injury is tissue specificity (15). Since most proteins, including H-FABP and myoglobin, are not specific for heart muscle, an attempt was made to find a solution to this problem. Since the tissue contents of myoglobin are higher in skeletal muscle than in heart, and the contents of H-FABP show the opposite, we hypothesized that the ratio of myoglobin over H-FABP in blood plasma after heart tissue injury is different from that after skeletal muscle injury. **Chapter 8** describes the use of the ratio of myoglobin to H-FABP in blood to discriminate between heart and skeletal muscle injury.

Finally, a general discussion about the implications of the results obtained in the present study for the mechanism of FA-uptake and oxidation in cardiomyocytes and for the use of H-FABP as a marker for muscle tissue injury is provided in **chapter 9**.

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CHAPTER 2

ROLE OF FATTY ACID-BINDING PROTEINS IN HEART FATTY ACID METABOLISM:

A short introduction

Parts of this chapter will be published in :
Van Nieuwenhoven FA, Van der Vusse GJ and Glatz JFC (1996): Membrane-associated and cytoplasmic fatty acid-binding proteins. Lipids, in press.

INTRODUCTION

Long chain fatty acids (FA) are a set of compounds consisting of a hydrophobic chain of hydrocarbons and a hydrophilic head group formed by carboxylic acid. In organisms, FA normally contain an even number of carbon atoms, varying from 14 to 24. The hydrocarbon chain is usually unbranched and can contain one ore more double bonds (86). FA are building blocks of triacylglycerols, phospholipids and glycolipids and they also serve as important fuel for energy conversion in a host of tissues including heart (14,15,94). In addition, FA were shown to be involved in certain signal transduction pathways (5,6,26,32,92), and can also be covalently bound to proteins, thereby influencing the biological role of these proteins (17,44,45,52,61).

Under physiological conditions the oxidation of FA in heart generates 30-70% of the energy required for the contraction-relaxation cycle, ion pumps and other important energy-consuming cellular processes (47,94). Since cardiac FA-synthesis is low, most of the FA used for oxidation and formation of complex lipids in the cardiomyocytes have to be derived from the blood (94). However, FA are poorly soluble in water and the rapid transport and metabolism of these FA would probably not be possible without special binding proteins, which substantially increase the solubility of these compounds in aqueous environments (97).

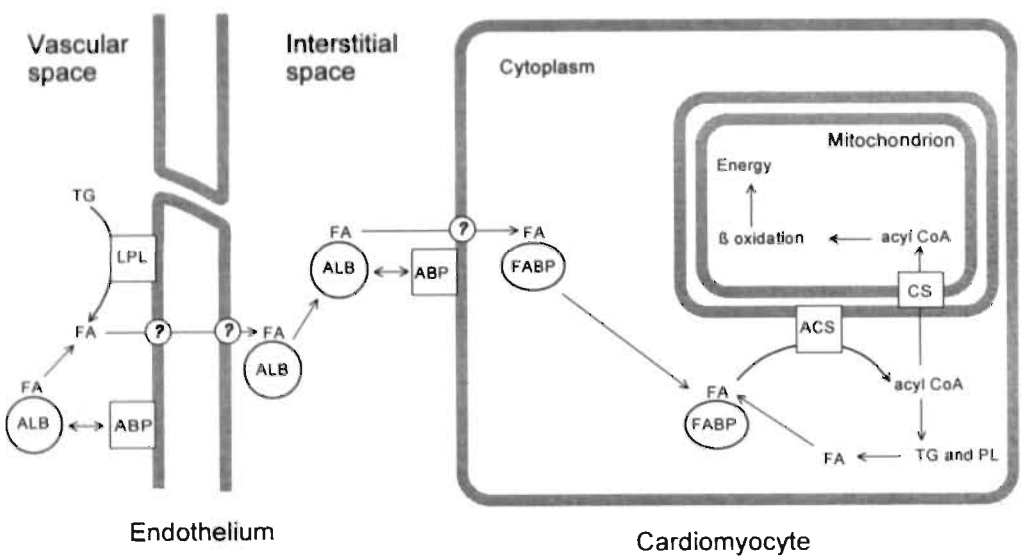

Figure 2.1 : *Schematic presentation of the transport and metabolism of FA in cardiac tissue. FA, long chain fatty acid; TG, triacylglycerol; ALB, Albumin; ABP, albumin binding protein; FABP, heart-type fatty acid-binding protein; ACS, acyl-CoA synthetase; PL, phospholipid; CS, carnitine shuttle, i.e. three enzymes involved in acyl transport across the mitochondrial inner membrane : carnitine acyltransferase I, carnitine acylcarnitine translocase and and carnitine acyltransferase II. ?, indicates that the transmembrane transport of FA is incompletely understood.*

Fig 2.1 shows a schematic presentation of the transport and metabolism of FA in the heart. In blood and interstitium, FA are mainly bound to albumin, which can bind up to 6 FA molecules (18,78,79). The albumin concentration in plasma is approximately 0.6 mM, while the FA concentration varies between 0.2 and 1 mM (78,94). The high affinity of albumin for FA raises their solubility in aqueous environments by several orders of magnitude while keeping the unbound FA concentrations in the nanomolar range (63,64,66). Intracellularly, FA are most likely bound to specific cytoplasmic fatty acid-binding proteins (FABP) (12,16,36,88,97). Membrane-associated proteins have been hypothesized to function in the cellular uptake of FA across the plasmamembrane of several cell-types including cardiomyocytes (83). These membrane and cytoplasmic proteins will be discussed in more detail below.

Once FA arrive at the outer membrane of the mitochondria they are linked to coenzyme A (CoA) by the enzyme acyl CoA synthetase. This energy-requiring reaction is driven by the accompanied degradation of ATP to AMP. Acyl CoA traverses the outer membrane of the mitochondria through pores, but it is not capable of passing the inner membrane of the mitochondria. At the inner side of the outermembrane of the mitochondria, the acyl group of acyl CoA is transferred to carnitine to form acyl carnitine by the enzyme carnitine acyltransferase I. This compound is transported across the innermembrane of the mitochondria by carnitine-acylcarnitine translocase, which shuttles carnitine out, and acyl-carnitine into the mitochondrial matrix. Once inside the mitochondria acyl carnitine together with CoA is reconverted into carnitine and acyl CoA. The latter reaction is catalyzed by carnitine fatty acyl transferase II. The activation of FA and transport inside the mitochondrial matrix is extensively reviewed in (94).

Inside the mitochondrial matrix, acyl CoA is degraded further via the ß-oxidation, yielding acetyl CoA, NADH and $FADH_2$ in each round. Acetyl CoA is oxidized in the citric acid cycle and the produced NADH and $FADH_2$ are oxidized in the respiratory chain pathway (86).

MEMBRANE ASSOCIATED FATTY ACID-BINDING PROTEINS

The mechanism of transmembrane transport of FA into parenchymal cells is still incompletely understood. Some investigators favour a mechanism in which FA cross the membrane by simple diffusion (24,25,67). Indeed, it was found that FA (at least the non-ionized acid form) can move relatively fast across phospholipid bilayers without the need for a transport protein (46). Others, however, found saturable uptake of FA by several cell-types, a process that could be inhibited by FA analogues, proteases and antibodies against specific proteins, together indicating that membrane-associated proteins are involved (3,4,77,82,83). Subsequently, by using various techniques a number of such membrane proteins have been identified (table 2.1).

The first membrane protein described to be involved in the uptake of FA is a 40 kDa protein present in the plasma membrane of rat liver cells (85). This protein, called $FABP_{PM}$, was subsequently also found in parenchymal cells of intestine (84) and in heart (76,77,83). The protein was found to be closely related, if not identical, to mitochondrial aspartate aminotransferase (87,108). $FABP_{PM}$ is not an integral membrane protein but has been shown to have affinity for cellular membranes (43,107,108). Recently, molecular biological studies showed that expression of $FABP_{PM}$ in different cell-types increased FA-uptake (28,43).

Table 2.1 : Membrane-associated fatty acid-binding proteins

Protein	Molecular mass (kDa)	Major occurrence	Reference
Membrane FABP	22	adipose tissue	(91)
$FABP_{PM}$	40	liver, heart, adipose tissue, intestine	(83,84)
FA receptor (FAR)	56-60	heart, kidney	(29,30)
FA transport protein (FATP)	63	adipose tissue, heart, skeletal muscle	(68)
FA translocase (FAT)	88	adipose tissue, heart, skeletal muscle	(1,41)

In 1987, Fujii and coworkers described another membrane protein with high affinity for FA, which was designated fatty acid receptor (29,30). This protein of about 60 kDa was found to be present in kidney and heart. At this time, further information on this protein is lacking.

The third membrane protein putatively involved in FA uptake, identified in adipocytes by Harmon and co-workers (40,41) and recently cloned by Abumrad et al. (1), is an 88 kDa protein called fatty acid translocase (FAT). FAT from rat was found to be highly homologous (85%) to the human leucocyte differentiation antigen CD36, a receptor protein present among others on monocytes and platelets, and thought to be involved in adhesion phenomena and intracellular signalling. For an extended review on the possible functions of CD36 the reader is referred to Greenwalt et al. (38). Comparison of rat FAT (1) and the recently cloned mouse CD36 (27) revealed an amino acid identity of 93%, which strongly suggests that these proteins are species homologues. Recent investigations showed that CD36 is an integral membrane protein with one transmembrane region (58). A schematic presentation of the possible structure of FAT is provided in fig 2.2.

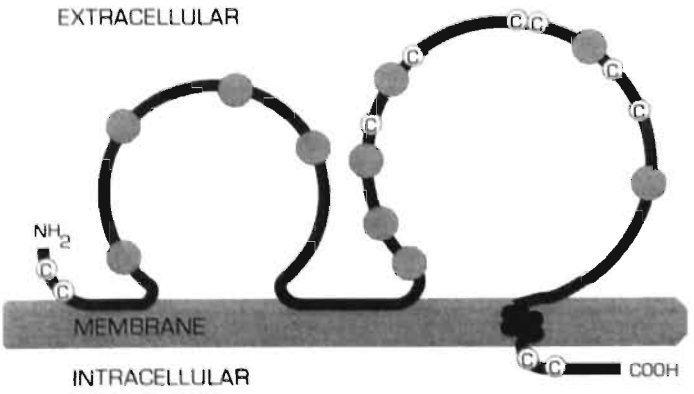

Figure 2.2 : *Schematic presentation of the possible structure and membrane orientation of putative fatty acid translocase (CD36). This structure is derived from ref (38), and modified using the additional information from ref (58). Closed circles indicate possible glycosylation sites and* **C** *indicates cysteine residues.*

In adipocytes, Trigatti et al. (31,91) found another membrane protein implicated in the transmembrane transport of FA. This 22 kDa protein was identified by photoaffinity labelling with a FA analogue.

The final membrane protein hypothesized to augment cellular FA uptake that has been found up till now, is named fatty acid-transport protein (FATP) (68). A functional approach using expression cloning was followed to identify this 63 kDa protein in adipocytes. FATP was shown to be an integral membrane protein present in the plasma membrane of several tissues, and has been predicted to have several membrane spanning domains (68). Considering the molecular masses of FATP and the fatty acid receptor found by Fujii and coworkers (29,30), it is possible that both proteins are related, if not the same.

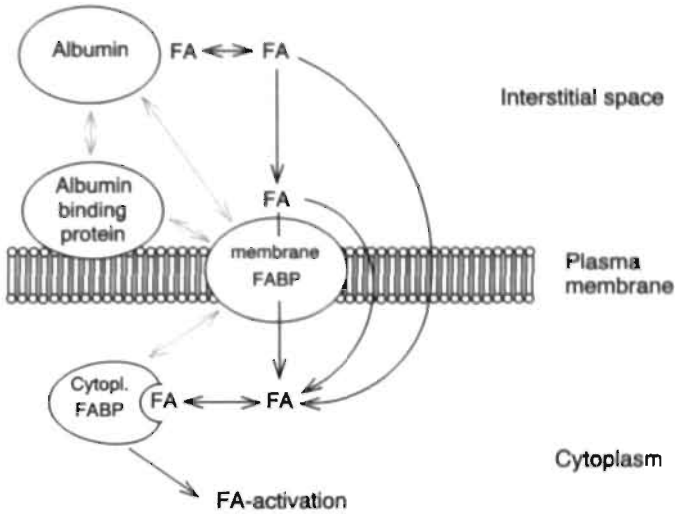

Figure 2.3 : *Schematic presentation of the putative mechanisms of cellular fatty acid uptake and the possible role of membrane-associated and cytoplasmic fatty acid-binding proteins in this process. Transmembrane transport could take place without the involvement of membrane proteins, or with a membrane protein acting as an acceptor for fatty acids thus creating a steeper transmembrane gradient and/or acting as a true FA translocator. FA, long chain fatty acid; membrane FABP, any membrane-associated fatty acid-binding protein (see table 1); cytopl. FABP, any cytoplasmic FABP (see table 2). Solid lines indicate possible routes of FA transport while the broken lines indicate possible protein-protein interactions.*

The precise functions of the FA-binding membrane-associated proteins in the transport of FA across cellular membranes are still unclear. The possible mechanisms by which membrane proteins could be involved in this process are shown in fig 2.3. Membrane associated fatty acid-binding proteins might function as a FA-translocator, but it is also possible that they represent acceptors for FA released from albumin and that FA subsequently cross the plasma membrane by diffusion through the phospholipid bilayer. Albumin binding proteins have been described which could play an additional role in this mechanism (9,10,60,70-72). Furthermore, there might be direct interaction between any of the membrane-associated FA-binding proteins and (extracellular) albumin and/or (intracellular) FABP. Such protein-protein interactions would ensure a controlled uptake mechanism in which FA is passed on from albumin to intracellular FABP but remains

protein-bound. Recently, Spitsberg et al. (80) found an association of H-FABP and FAT in the bovine mammary gland. These authors describe an interaction of the cytoplasmic site of FAT with the H-FABP, which could be involved in the FA-uptake across the membrane (80).

Some investigators found the FA-uptake to depend upon the non-protein bound ('free') FA-concentration (67,76), which makes the involvement of albumin binding proteins and the interaction between albumin and membrane-associated proteins debatable. In conflict with these findings is the recent report of a direct role for albumin in the cellular uptake of FA (90). Further characterization of the FA-binding membrane proteins will provide a more detailed understanding of the transmembrane transport mechanisms of FA.

INTRACELLULAR FATTY ACID-BINDING PROTEINS

The first reports of a fatty acid-binding protein in the cytoplasm of several cell-types were published in 1972 (51,55). These researchers incubated cytosolic preparations of liver, intestine, heart, skeletal muscle, adipose tissue and kidney with radio-labelled oleate. Thereafter, gel permeation chromatography was performed and radioactivity was found to coelute with a low molecular weight protein which was designated "fatty acid-binding protein" (FABP). In the following years, these FABP were purified and characterized, and distinct types of FABP were described. It was found that cytoplasmic fatty acid-binding proteins (FABPs) belong to a gene family of intracellular lipid binding proteins (LBP) of 14-15 kDa, capable of binding hydrophobic ligands with high affinity (13). The mammalian cytoplasmic lipid-binding proteins described until today are listed in table 2.2.

Since the first reports in 1972, thirteen different lipid-binding proteins, including the recently discovered testicular 15 kDa protein (T-LBP) (56,69), have been identified (table 2.2). Each of the lipid binding proteins is composed of 2 α-helices and 10 anti-parallel ß-strands, organized in two ß-sheets, thereby forming a clam shell-like structure (12). The lipid ligand is bound inside the molecule by interaction with specific amino acid residues within the binding pocket of the lipid binding protein (12). A recent investigation by Richieri et al. (65) suggests that the FABPs have a higher affinity for the binding of FA than found in previous studies (16,36,97), with the dissociation constants (Kd) ranging from 2 to 1000 nM, depending on type of FABP and of FA (65). The methods used in earlier studies to measure the FA-binding to FABPs involved the physical separation of free and protein-bound FA (57,96,100). This separation can influence the binding of FA to FABP because a new equilibrium will be formed after the separation. In this way the "FABP bound" FA fraction is underestimated and thus the dissociation constant is overestimated (65). A striking feature of the FABPs is their relative abundance in tissues with active FA metabolism. In itself this is a strong indication that the roles of the different FABP-types have to be found in the FA-metabolism in these tissues. A number of biological roles have been ascribed to the FABPs:

1) Facilitation of the transport of FA to their intracellular sites of utilization. Indirect evidence was provided in studies showing a correlation between FA-utilization and FABP-content (34,98), and by showing that *in vivo* FA are bound to FABP (103,104). Furthermore, *in vitro* studies showed an increase in FA transport from isolated mitochondria to artificial phospholipid vesicles in the presence of H- or L-FABP (59). It was shown that the transfer of FA from H-FABP and L-FABP to model phospholipid membranes occurs via different mechanisms (48,49), indicating different functions in FA

Table 2.2 : Mammalian cytoplasmic lipid binding proteins. Data a[1] compiled from refs (12,32,50,56,69)

Protein	Current designation	Other designation	Ligand	Major occurrence
Heart FABP	H-FABP	MDGI	FA	Heart, skeletal muscle, smooth muscle, brain, kidney, mammary gland
Liver FABP	L-FABP	Z-protein	FA, heme, bilirubin, prostaglandins	Liver, small intestine, kidney
Intestinal FABP	I-FABP		FA	Small intestine
Adipocyte lipid-binding protein	A-LBP	A-FABP, a[2]	FA, retinoic acid	Adipose tissue
Epidermal FABP	E-FABP		FA	Epidermis
Brain FABP	B-FABP		FA	Nervous system
Myelin lipid-binding protein	M-LBP	myelin-P2	FA, retinoids	Nervous system
Ileal lipid-binding protein	I-LBP	I-BABP, gastrotropir	FA, bile acids	Small intestine (ileum)
Testicular lipid-binding protein	T-LBP	PERF15	not determined	Testis
Cellular retinoic acid-binding protein	cRABP		retinoic acid	Testis, nervous system, kidney, skin
Cellular retinoic acid-binding protein II	cRABPII		retinoic acid	Skin, adrenals
Cellular retinol-binding protein	cRBP		retinol	widespread expression
Cellular retinol-binding protein II	cRBPII		retinol	intestine

metabolism. The mechanism of FA transfer from A-FABP to membranes was found to be similar to that of H-FABP (105,106), which may relate to the high degree of sequence homology between these proteins (62%). Stewart et al. (81) reported a H-FABP-facilitated diffusion of oleate in a model cytosol system, but this conclusion was disputed by others arguing that appropriate control experiments were lacking (101). Theoretical studies also support a role for H-FABP in FA-transport in cardiomyocytes (102). More direct evidence was found by molecular biological studies in which cells transfected with H-, L- or A-FABP showed increased FA-uptake rates (22,73,74). In contrast to these three types of FABP, transfection of L-cell fibroblasts with I-FABP did not increase the initial rate and extent of FA uptake (62). Expression of I-FABP increased the incorporation of FA into triacylglycerols, again showing the different roles of distinct types of FABP (62). Finally, a recent report showed that a single amino acid substitution (Ala54 -> Thr54) in human I-FABP, as occurs in some Pima Indians, causes a two-fold greater affinity for FA *in vitro*. This was associated with increased fat oxidation rates *in vivo*, suggesting a role for I-FABP in the absorption and processing of FA in the intestine (11).

2) Prevention of local high FA concentrations and thereby protecting the cell against detrimental effects of FA, for instance in the ischemic heart (34,37). During ischemia, there is an increase in FA concentration in the heart due to an impaired ß-oxidation and enhanced phospholipid degradation (94). These high concentrations of FA, which are partly incorporated into membranes, can influence membrane stability and eventually lead to disruption of the membrane (94). By competing with membranes for the binding of FA, FABP could prevent this destructive effect. However, the calculations of Vork et al. (102) on the distribution of FA between the sarcolemma and H-FABP show that under physiological conditions only about 13% of FA is bound to H-FABP. Since ischemia most probably does not alter the partition coefficient of FA between membranes and H-FABP, a major role for H-FABP in preventing high FA concentrations in the membrane remains to be established.

3) By modulating hydrophobic ligand metabolism, FABPs can participate in important cellular events like mitogenesis (75) and FA-mediated signal transduction pathways (32,36). Recently, a small fraction of H-FABP (<1%) in rat heart and in mammary gland was found to be phosphorylated upon stimulation with insulin, and it was suggested that H-FABP might play a role in signal transduction downstream from the insulin receptor (53,54). In vitro phosphorylation of the A-FABP by the insulin receptor was reported earlier by Buelt et al. (19).

4) Inhibition of growth and induction of differentiation of cultured mammary epithelial cells has been reported as an extracellular function of mammary derived growth inhibitor (MDGI), which is identical to H-FABP (16).

5) Induction of myocyte hypertrophy through binding with a high affinity receptor is another extracellular function recently ascribed to H-FABP (20).

REGULATION OF EXPRESSION OF FAT AND H-FABP

Since the studies described in this thesis handle the roles of H-FABP and FAT in FA uptake and metabolism in the heart, the regulation of expression of these proteins will be set out in more detail. H-FABP is known to be present in a number of tissues (table 2.2), with high expression levels found in heart, skeletal muscle and lactating mammary gland (97). H-FABP expression in rat heart increases rapidly after birth, to reach levels

of about 2% of all cytosolic proteins at adulthood (23,42,95,99). This increase is parallelled by a postnatal increase in palmitate oxidation capacity of the heart tissue (35). In skeletal muscle the expression of H-FABP is related to the content of type 1 (oxidative) muscle fibers, with a maximal expression of about half of that found in heart (99). Recent investigations showed that the H-FABP expression in rat heart and skeletal muscles was elevated during experimental diabetes and fasting (21,33). Both interventions are thought to increase the FA utilization of heart and skeletal muscle. In addition, the H-FABP content of rat heart and skeletal muscles was also increased upon endurance training and testosterone treatment (93).

A coexpression was found to exist of CD36 (which is FAT) and H-FABP in bovine mammary gland (80). Expression of both proteins was related to mammary cell differentiation and was highest during lactation (80). Coexpression of H-FABP and FAT was found earlier by our group to exist in rat muscle tissues (95), and is described in chapter 4.

Expression of FAT mRNA was found to be abundant in heart, intestine, fat, skeletal muscle and testis (1,95). The presence of this protein in heart tissue was confirmed by Tanaka and Kawamura (89). In cultured preadipocytes (Ob1771, 3T3F442A) FAT expression is upregulated during differentiation from preadipocytes to adipocytes (1), and is parallelled by an increased uptake of FA (2). Recently it was shown that FA themselves can induce the expression of FAT in Ob1771 preadipocytes (7). It was hypothesized that a protein, related to the peroxisomal proliferator-activated receptors (PPAR) and called fatty acid-activated receptor (FAAR), mediates these transcriptional effects of FA in preadipocytes (8).

In conformity with the increase of H-FABP in experimental diabetes in the rat (33), CD36 (FAT) expression was found to be elevated in murine models of diabetes (39). An increase in CD36 expression was also observed after a high fat diet, indicating that the expression of this protein is related to lipid metabolism (39). In this thesis, some preliminary studies show that also in the rat, experimental diabetes tends to increase FAT expression in the heart (chapter 4).

CONCLUDING REMARKS

During the last two decades it has become clear that a number of non-enzymatic proteins are involved in the metabolism of FA in the heart. The precise roles of membrane-associated FA-binding proteins and the intracellular H-FABP in uptake, transport and metabolism of FA in heart, however, remain to be established. In the next three chapters of this thesis some aspects of FA-uptake in the myocardium will be discussed in more detail.

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CHAPTER 3

FATTY ACID TRANSFER ACROSS THE MYOCARDIAL CAPILLARY WALL:

No evidence of a substantial role for cytoplasmic fatty acid-binding protein

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ABSTRACT

It has recently been hypothesized that fatty acid (FA) transfer across the myocardial capillary wall is mediated by cytoplasmic fatty acid-binding protein (FABP). Therefore, we studied the type and content of FABP in endothelial cells from rat heart, using molecular biological, immunochemical, and FA-binding assays. Studies were performed on short term cultured endothelial cells, two established endothelial cell lines and ultrathin cryosections from adult rat heart.

Northern blotting analysis of endothelial cell RNA failed to detect either heart-type (H-) FABP or liver-type (L-) FABP mRNA, but the reversed transcription-polymerase chain reaction revealed both H- and L-FABP mRNAs, indicating the presence of minor amounts of these mRNAs. Highly sensitive immunochemical assays (sandwich ELISAs) using specific antibodies raised against rat H- or L-FABP showed the contents of these FABP-types in endothelial cells to be 1-5 ng/mg cytosolic protein, which is more than 3 orders of magnitude lower than the contents of H-FABP in heart or L-FABP in liver. Immuno-electron microscopy also showed that the concentration of H-FABP in endothelial cells is at least 2 orders of magnitude lower than that in cardiomyocytes. Finally, cytosolic protein samples from endothelial cells revealed no significant FA-binding activity in the 15-kDa region.

We conclude that rat heart endothelial cells contain only minor quantities of cytoplasmic FABP and that, therefore, FA transport over the endothelium is mediated by FABP only to a minor extent. It is postulated that aqueous diffusion of FA through the endothelial cytoplasm most likely accounts for the experimentally observed rates of cardiac FA utilization.

INTRODUCTION

Oxidation of long chain fatty acids (FA) is a major source of energy production in the heart (5,34). Because FA are poorly soluble in an aqueous environment, special mechanisms are needed to provide the tissue cells with appropriate amounts of FA (3). In blood and interstitium, FA are bound to albumin, thereby raising their solubility several orders of magnitude (30).

Cardiomyocytes contain relatively large amounts of a cytoplasmic fatty acid-binding protein (FABP) (21). FABPs are 15 kDa proteins present in a number of mammalian tissues and capable of binding FA. To date several types of FABP have been identified, of which only the heart type (H-) and the liver type (L-) FABP are present in more than one tissue. Other FABP-types from intestine, adipose tissue, brain and skin appear to be tissue specific (10). Earlier studies have shown that FABPs are likely to play an important role in the intracellular translocation of long chain FA (37,39).

In the heart, endothelial cells form the first barrier blood-borne FA have to overcome before they can be metabolized in the underlying cardiomyocytes (3,32). It has been hypothesized that, like in the cardiomyocytes, FABP facilitates the diffusion of FA through the endothelial cytoplasm (3,9). Several authors have found H-FABP to be present in endothelial cells (9,18,22,23,40). The actual content of H-FABP in these cells, however, is still a matter of debate (34).

The aim of the present study was to quantify H-FABP and/or L-FABP in endothelial cells of adult rat heart, and to study the presence, if any, of other cytoplasmic proteins

with FA-binding properties. For this we performed molecular biological, immunochemical as well as FA-binding assays, using isolated rat heart endothelial cells (either short term culture or established cell-lines) as model systems (17). In addition, immuno electron microscopy was performed using ultrathin cryosections of intact adult rat heart.

The molecular biological studies consisted of northern blotting analysis of endothelial cell RNA with H-FABP and L-FABP cDNA as probes. Furthermore, reversed transcription-polymerase chain reaction (RT-PCR) was performed with primers for H-FABP and L-FABP. The protein content of H-FABP and L-FABP in endothelial cells was determined using indirect non-competitive sandwich ELISAs as described by Vork et al. (35). A new sandwich ELISA for L-FABP from rat was developed for this purpose. To exclude effects of culturing of cells on the expression of H-FABP or L-FABP we also applied the specific antibodies against these two proteins in a semi-quantitative immuno-gold labelling procedure on cryosections of rat heart using electron microscopy.

To study the presence of other, known or unknown cytosolic proteins capable of non-covalently binding FA in the endothelial cells, we developed an assay for FA-binding activity. This assay was modified from that of Samanta et al. (25), and is based on the ability of the FABP to bind with high affinity radiolabelled long chain FA.

The present findings show that in rat heart endothelial cells both H-FABP and L-FABP are present in very low quantities. Furthermore, no indications could be found for the presence of other cytoplasmic 15 kDa proteins with appreciable FA-binding capacity within these cells. The consequence of the present findings for the mechanism of trans-endothelial FA-transport is discussed.

MATERIALS AND METHODS

Cell culture techniques

Cells were isolated from hearts of adult male Wistar or Lewis rats as described by Linssen et al. (16-18). The cardiomyocytes were microscopically checked for cell morphology and trypan blue exclusion. More than 95% of the cells were rod shaped and excluded trypan blue.

The primary cultures of endothelial cells were tested for the uptake of Acetylated Low-Density Lipoprotein labelled with 1,1'-dioctadecyl- 1-3,3,3',3'- tetramethyl-indocarbocyanine-perchlorate (DiI-Ac-LDL). Endothelial cells were also tested for the presence of Von Willebrand Factor (FVIII/VWF) and of a special endothelial cell membrane antigen, using a specific antibody against rat endothelial cell membrane (RECA1).

In the present study we used endothelial cells in passages one through four, as well as two immortalized cell-lines of adult rat heart endothelial cells, designated RHEC-50 and RHEC-116, as described by Linssen et al. (17). Routinely, these cells were cultured in endothelial culture medium containing 40% M199, 40% RPMI1640, 20% FCS, supplemented with 2 mM L-glutamine, 13 mM $NaHCO_3$, 10 mM HEPES, 50 μg/ml gentamicin, 20 U/ml heparine and 2 ml/l growth factor prepared from bovine brain. Media and supplements were obtained from Gibco BRL, Life Technologies (Gaithersburg, MD, USA) except for FCS which was derived from SEBAK (Aidenbach, Germany).

To investigate the effect of exogenous FA on FABP expression, RHEC-116 was cultured in FA-rich, glucose-poor medium. Culture media used to test the possible effect of FA was glucose free RPMI1640, containing 20% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, 20 U/ml heparin and 2 ml/l growth factor prepared from bovine brain. After

oleic acid was complexed to bovine serum albumin (BSA) as described by Brodersen et al. (6), media were prepared containing 0.6 mM BSA and 0, 0.3 or 1.2 mM oleic acid. Thereafter, RHEC-116 was grown on each of the media until confluency. After the cells had been confluent for at least one day they were harvested and the H-FABP and L-FABP contents assayed.

RNA isolation

Rat tissues used for RNA isolation were dissected from 10-weeks old female Lewis rats, immediately frozen in liquid nitrogen and stored at -80°C until use. In all steps of the procedure 0.1% diethyl pyrocarbonate (DEPC) treated water was used (26). RNA was isolated according to Chomczynski & Sacchi (7). Rat tissue (ca. 100 mg) or 10^8 cells were homogenized in 1 ml guanidinium solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol). Subsequently, 0.1 ml of 0.1 M sodium acetate (pH 4), 1 ml phenol saturated with water and 0.2 ml chloroform-isoamylalcohol (49:1, v/v) were added. After centrifugation at 10,000 g the water fase was transferred to a fresh tube and mixed with 1 ml isopropanol to precipitate RNA (1 h at -20°C). After centrifugation at 10,000 g for 20 min the pellet was dissolved in guanidinium solution and precipitated again with an equal volume isopropanol for 1 h or overnight at -20°C. After centrifugation the pellet was washed with 70 % ethanol, dissolved in DEPC treated water for direct use or stored at -80°C in 70% ethanol. Poly-A RNA was isolated from the RNA preparations using the poly-A quick mRNA purification kit of Stratagene (La Jolla, CA, USA), according to the instructions of the manufacturer. On the average 6 μg poly-A RNA were isolated from 300 μg RNA.

Northern blotting

H-FABP (13), L-FABP (12) and GAPDH cDNAs were used as probes in the Northern blot analyses. RNA samples were dissolved in sample buffer containing 20 mM [morpholino]ethane sulfonic acid buffer (MOPS, pH 7), 6.6% formaldehyde, 50% formamide and 0.5% ethidium bromide, and subjected to electrophoresis on a formaldehyde and MOPS containing 1.5% agarose gel. Ethidium bromide was added to monitor the RNA separation during the electroforesis. RNA was transferred to nitrocellulose (BA85, Schleicher and Schull, Einbeck, Germany) overnight by capillary action in 10 times concentrated saline sodium citrate buffer (10 x SSC; 1.5 M NaCl, 0.17 M $Na_3C_6H_5O_7$, pH 7.0).

cDNA probes were labelled radioactive using the Random Primers Labelling System (Gibco, Life Technologies, Gaitersburg, MD, USA). Filters were prehybridyzed for one hour at 60°C in 0.5 M Na_2PO_4, pH 7.0 containing 7% SDS, 1% BSA, 2 mM EDTA and 1 mg/ml sonicated salmon sperm DNA, and thereafter were incubated with the cDNA probes overnight at 60°C. Filters were washed at 60°C successively in: 3 x SSC for 1 h; 1 x SSC for 1 h; 0.1 x SSC + 0.1% SDS for 15 min, and exposed to X-ray film at -70°C using intensifying screens.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Oligo-nucleotides were synthesized by Eurogentec (Liege, Belgium). Superscript reverse transcriptase, Taq DNA polymerase and restriction enzymes were purchased from Gibco, Life Technologies (Gaitersburg, MD, USA).

First strand synthesis was performed 1 hr at 37°C with 10 μg of total RNA using 1 μl (200 units) Reverse Transcriptase in a final volume of 20 μl. PCR was carried out as

described by Sambrook et al. (26), using 2 μl of the reversed transcriptase product in a final volume of 50 μl, and a $MgCl_2$ concentration of 2.0 mM. PCR was performed according to the following scheme: 5 min denaturation at 94°C; 35 cycles of 1.5 min at 94°C, 1.5 min at 53°C, 2 min at 72°C; and finally 5 min at 72°C.

Table 3.1 : *Specific and degenerated primers for H- and/or L-FABP, as used in RT-PCR. 5H and 3H are specific primers for H-FABP, 5L and 3L are specific primers for L-FABP. 5N and 3N are nested primers homologous with both H- and L-FABP. Capital characters represent sequences homologous with H- and/or L-FABP. Small characters are sequences, containing restriction sites, included for purpose of cloning. Bold characters represent the restriction sites for cloning into pUC-19. For the nested primers (5N and 3N) the degenerated bases are presented as super- and subscript characters. The superscript characters show the sequence homologous with H-FABP while the subscript characters show the degenerated bases according to the L-FABP sequence. -, not present in L-FABP sequence.*

Primer		Sequence	Product-size
5H	H-FABP	ttc**aagcTT**GCACCATGGCGGACGCCTTT	435 bp
3H	H-FABP	AGTGACGGG**GgAtCC**AGGTCACGCCTCCTT	
5L	L-FABP	ttc**aagctt**ATGAACTTCTCCGGCAAGTAC	402 bp
3L	L-FABP	ctg**ggatcc**CTAAATTCTCTTGCTGACTCTCTT	
5N	H/L-FABP	atcggatcc**gaattc**A^{T} TTTGA$_{C}$ CT^{TGA} CATGAAG$_{GCC}$ ^{A} $_{T}$	363 bp on H-FABP 344 bp on L-FABP
3N	H/L-FABP	cgaagctt**tctaga**CGA^{G} TG^{C} TCACC^{A} CA^{T} TG^{C} CATGG$_{-}$ $_{-}$ $_{-}$ $_{G}$ $_{T}$	

Primers used in the PCR reaction are shown in table 3.1. The 5H and 3H-primers are homologous to the 5' and the 3' H-FABP cDNA coding region. The 5L and 3L-primers are homologous to the 5' and the 3' L-FABP cDNA coding region. The sequences of the nested primers were the same for both H-FABP and L- FABP since they were selected on the basis of homology between the two genes. Because no region with 100% homology was found, degenerations were included (see table 3.1). Sequences coding for the restriction sides BamHI, EcoRI, HindIII and XbaI were added to the primers to enable cloning of the PCR fragments into the pUC-19 vector. Fragments obtained by PCR were analyzed by restriction analysis (H-FABP and L-FABP) and DNA-sequencing (L-FABP).

Preparation of tissue and cell homogenates.

Rat heart and liver tissues and isolated cell homogenates as used in the immunoassays and the FA-binding assay were prepared as follows. Hearts from male Lewis rats were perfused according to Langendorff, exactly as described in an earlier study by Vork et al. (36), so as to remove blood and interstitial constituents. During the 5-hr period of

normoxic perfusion no more then 1.1% of total tissue protein was lost in the effluents (36). Likewise, livers from male Lewis rats were perfused thoroughly in situ with phosphate buffered saline (PBS) to remove blood from the vascular compartment.

All following steps were performed on ice or at 4°C. Both heart and liver tissues were cut and homogenized (2-10%, w/v) in PBS using an Ultra-Turrax homogenizer (IKA Werke, Breisgau, Germany). The homogenates were sonicated 4 times for 15 s at 12 microns, and centrifugated at 1,500 g for 15 min. Cultured cells were harvested using trypsin/EDTA treatment, or using a rubber policeman, sonicated 4 times for 15 s at 12 microns, and centrifugated at 10,000 g. Cells and tissue homogenates were then centrifugated at 105,000 g for 90 min to obtain a 105,000 g supernatant (referred to as cytosol). Cell and tissue supernatants were stored at -80°C until use. Protein concentration was measured according to Lowry et al. (20).

Immunoassays for H-FABP and L-FABP

Microtiter plates were obtained from Becton Dickinson (Oxnard, CA, USA), Protein A Sepharose and CNBr-activated Sepharose from Pharmacia (Uppsala, Sweden), Streptavidin conjugated with horse-radish peroxidase (Streptavidin-HRP) from Pierce Chemical Co. (Rockford, IL, USA), ortho-phenylene diamine (OPD) and bovine serum albumin (BSA, type A7888) from Sigma (St. Louis, MO, USA), and Tween-20 from Merck (Darmstadt, Germany).

Rat H-FABP was measured using an indirect non-competitive sandwich ELISA as described by Vork et al. (35). For assaying rat L-FABP a novel sandwich ELISA was developed using a similar procedure. In short, antibodies against recombinant L-FABP were raised in rabbits. The antibodies were purified using affinity chromatography on a Protein A sepharose column and subsequently on a column on which partially purified L-FABP was covalently bound to CNBr-activated sepharose. After affinity purification part of the amount of antibodies was biotinylated. Recombinant L-FABP (a gift from dr. D.P. Cistola, St. Louis, USA) was used as standard in the assay. For the ELISA procedure, 96-wells microtiter plates were coated with antibody (250 ng/well) in 0.1 M carbonate buffer pH 9.6 for 2 hr at 37°C. Between consecutive steps the plates were washed 4 times with PBT (PBS containing 0.1% BSA and 0.05% Tween 20). Samples and standards were incubated overnight at 4°C. Then 400 ng of biotinylated IgG was added to each well and incubated for 90 min at room temperature. Thereafter streptavidin-HRP was added to the wells and incubated for 1 hr. The plate was developed using 100 μl OPD/H_2O_2 solution. Development was stopped with 50 μl 2M H_2SO_4, and the extinction was determined at 492 nm in a Titertek Multiskan MKII (Flow Laboratories, Lugano, Switzerland).

The sandwich ELISA for L-FABP was found to be comparable with the assay for H-FABP with respect to detection limit, recovery and reproducibility (35). We found the detection limit to be 0.5 μg/l, which corresponds with 25 pg/well. The recovery of added pure L-FABP to homogenates was 96 ± 10% (mean ± SD of 6 experiments), the intra-assay variation 6% and the interassay variation 11%. The cross-reactivities of H-FABP in the L-FABP-assay, and conversely, were found to be below 0.002%.

Immunohistochemical detection of FABP

Hearts of ether anaesthetized adult male Wistar rats were quickly excised, and small fragments of the tissue were fixed using a 0.1 M sodium phosphate buffer (pH 7.4) containing 2% paraformaldehyde and 0.2% glutaraldehyde. Ultracryotomy was performed

using a slightly modified Tokuyasu procedure (31) as described in detail by Slot et al. (29). Tissue fragments were washed in phosphate buffered saline (PBS) for five minutes and immersed in 2.3 M sucrose in PBS for one hour. Tissue blocks were mounted on specimen holders and frozen in liquid nitrogen. For electron microscopy (EM), ultrathin (ca. 100 nm) sections were cut at -120°C with a glass knife. Sections were picked up, thawed and subsequently transferred to copper grids. Immunolabelling of H- or L-FABP were performed using specific affinity purified rabbit antibodies against these proteins (see above). Grids, containing the cryosections were floated at room temperature on successive drops of the following solutions: (a) 0.02 M glycine/PBS (3x10 min); (b) PBS/1% BSA (2x5 min); (c) affinity purified rabbit anti H- or L-FABP IgG (30 min); (d) biotin-labelled donkey anti rabbit IgG (30 min); (e) streptavidin-gold (10 nm) (30 min). Control sections were incubated on drops of PBS without the primary antibodies, and were treated identically thereafter. The cryosections were stained on drops of uranyl acetate-oxalate, washed with PBS and embedded in methyl cellulose. The sections were examined using a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Semiquantitative analyses were performed on preparations from four animals according to Fournier and Rahim (9), by counting the number of dots (representing goldparticles) per square μm on a digitizer (Summagraphics ID, Fairfield, CT, USA).

Assay of FA-binding activity

A Superdex 75 HR 10/30 column was purchased from Pharmacia (Uppsala, Sweden) and calibration proteins from Sigma (St. Louis, MO, USA). Lipidex 1000 and Ultima Flow M were obtained from Packard Instrument Company (Meriden, CT, USA). Radiolabeled 9,10-[^{3}H]-palmitic acid (specific activity 54 kCi/mol) was obtained from Amersham Int. (Amersham, UK). Immediately before use, palmitic acid was checked for purity using thin layer chromatography.

The HPLC system used (Varian, CA, USA) consisted of a HPLC pump (type 2510), a Valco injector with a sample loop of 100 μl and a variable wavelength detector (type 2550). In addition, an on line radio-chromatography detector A-250 (Radiometric Instruments & Chemical Co., Meriden, CT, USA) was used, existing of a flow-through cell between photomultiplier tubes for radioactive detection by the scintillation method. In this detector the eluate is automatically mixed with scintillation reagent in a mixing tee. The delay between the signal from the wavelength detector and the radio-chromatography detector was 36 s.

The assay of FA-binding activity consisted of a High Performance Gel Permeation Chromatography (HPGPC) technique, combined with direct detection of FA-binding capacity using radioactive FA. 150 μl of 105,000 g supernatants from heart, liver or cell-homogenates were mixed with 3-10 μl of radiolabeled FA solution (corresponding with 3-10 μCi), incubated for 10 min at 37°C and centrifuged at 10,000 g for 10 min. Then 100 μl of the supernatant was injected onto a Superdex-75 HPGPC column. Protein was measured at 280 nm by a wavelength detector. Radioactivity was measured using an on-line radiochromatography detector. In a number of cases the samples were delipidated beforehand. For this, 200 μl of the 105,000 g supernatants were incubated for 20 min at 37°C with 100 μl of Lipidex 1000 (11). After delipidation, the mixture was centrifuged at 10,000 g for 2 min. Thereafter, 150 μl of the supernatant was mixed with 10 μl of radiolabeled FA solution and treated exactly as described above.

RESULTS

Northern blotting

Northern blots of RNA from rat heart, liver and RHEC-116 hybridized with cDNAs of H-FABP, L-FABP and GAPDH are presented in fig 3.1. GAPDH was detectable in each RNA sample studied, but for liver RNA only after prolonged exposure. Ethidium bromide intercalation of RNA samples on the blot showed a clear signal for the lane with liver RNA. Hybridization with L-FABP cDNA gave a positive signal for this RNA sample, indicating that L-FABP RNA was present in the sample.

In liver only L-FABP, and in heart only H-FABP could be detected. RHEC-116 RNA showed no detectable hybridisation with either L-FABP cDNA or H-FABP cDNA as probe, even when 5 μg of poly-A RNA were applied to the gel (fig 3.1). Northern blotting analysis with RHEC-50 RNA showed results similar to those found for RHEC-116 (data not shown).

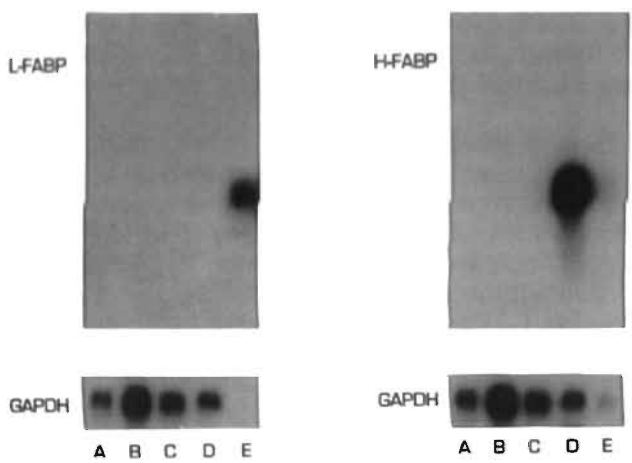

Figure 3.1 : *Northern blot analysis of rat heart, liver and RHEC-116 RNA or poly-A RNA probed with L-FABP cDNA (left panel), H-FABP cDNA (right panel) and GAPDH cDNA (bottom panels). Lane A: 15 μg RHEC-116 RNA, lane B: 5 μg RHEC-116 poly-A RNA, lane C: 1.5 μg RHEC-116 poly-A RNA, lane D: 7 μg heart RNA, lane E: 5 μg liver RNA.*

Reversed transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on RNA samples from rat heart, liver and the endothelial cell-lines RHEC-50 and RHEC-116. The primers used in the RT-PCR are shown in table 3.1. The results of the RT-PCR with specific primers and those of a second PCR with nested primers are shown in fig 3.2. With specific primers for L-FABP the PCR showed an intense band at about 400 bp with liver RNA and also with heart RNA. The specific primers for H-FABP yielded an intense band at about 430 bp with heart RNA and a weaker signal with liver RNA. In the subsequent nested PCR, products of about 350 bp were seen in all preparations studied (fig 3.2).

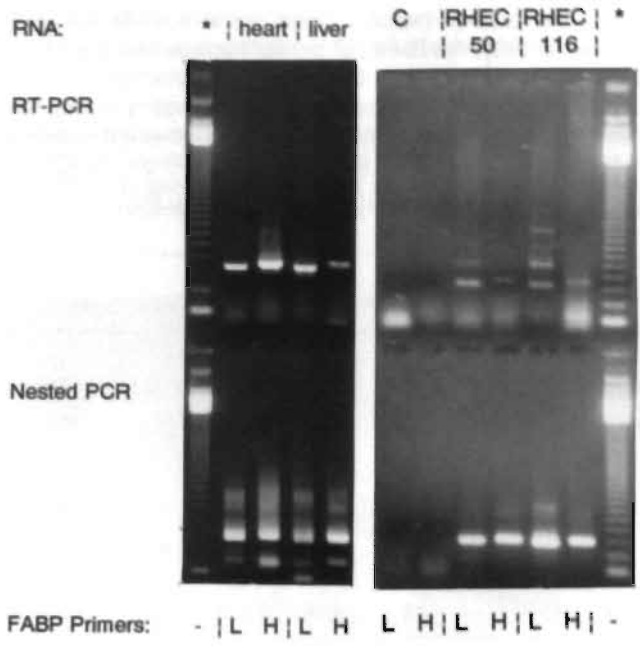

Figure 3.2 : *Analysis of RT-PCR products from rat heart, liver, RHEC-50 and RHEC-116 RNA using specific primers against H- and L-FABP, and the products of the nested PCR using the degenerated primers. L, specific primers for L-FABP; H, specific primers for H-FABP; *, 123-bp ladder of molecular size markers; C, negative control (distilled water).*

The results of RT-PCR of RNA from the endothelial cell-lines RHEC-50 and RHEC-116 show several bands, with the H-FABP as well as L-FABP specific primers. However, the nested PCR of these PCR samples yielded only those fragments corresponding to the sequences from H-FABP and L-FABP. The negative control gave no signal (fig 3.2). The positive reaction in the nested PCR confirms that the fragments obtained in the first PCR represented L-FABP and H-FABP. This was further verified by restriction analysis (H-FABP and L-FABP) and DNA sequencing (L-FABP) (data not shown).

Sandwich ELISA for rat H-FABP and L-FABP

The H-FABP and L-FABP contents measured in homogenates of rat heart, liver and cultured endothelial cells using the specific sandwich ELISAs for H-FABP and L-FABP are given in table 3.2. The heart tissue H-FABP content of 15,400 ng/mg cytosolic protein corresponds to 0.69 mg H-FABP per gram of total heart tissue which is in accordance with earlier reported FABP contents of rat heart (35). Rat liver contains 17,200 ng L-FABP/mg cytosolic protein, which corresponds to 1.8 mg L-FABP per gram of liver tissue. The small amounts of H-FABP in liver homogenates and L-FABP in heart homogenates as found with the sandwich ELISAs (table 3.2) are not due to immuno-chemical crossreactivity of these proteins (see Materials and Methods), but aspecific binding of the anti-FABP antibodies to other cellular cytosolic proteins can not be excluded. The detection of H-FABP in liver homogenates most likely relates to the

presence of smooth muscle cells in hepatic blood vessels walls, as in rat hepatocytes, isolated as described earlier (33), the H-FABP content amounted 2 and 3 ng/mg cytosolic protein (two preparations).

Table 3.2 : *Contents of H- and L-FABP in rat heart and liver tissue and in cultured rat heart endothelial cells as measured by specific immunochemical assays. H- and L-FABP were assayed with specific sandwich-ELISAs as described in Materials and Methods. Data are expressed as means ± SD of the indicated number of measurements.* *, *n=3.*

Preparation	n	H-FABP	L-FABP
		(ng/mg cytosolic protein)	
Heart homogenate	5	15,400 ± 1400	2 ± 1
Liver homogenate	6	9 ± 1	17,200 ± 300
Endothelial cells :			
Short term culture	13	5 ± 4	1 ± 1
Cell-line RHEC 50	8	3 ± 2	1 ± 1 *
Cell-line RHEC 116	23	3 ± 3	0.5 ± 0.2

In the endothelial cells the content of H-FABP was found to be 3-5 ng/mg cytosolic protein, which is in agreement with H-FABP contents documented in an earlier report from our laboratory (18). The L-FABP content of the endothelial cells was about 1 ng/mg cytosolic protein (table 3.2).

We studied the expression of FABP in RHEC-116 cultured in FA-rich, glucose-poor media. No differences were observed in the H- or L-FABP content of this established cell-line before, during and after several days of incubation with oleic acid, not even after several passages. The H-FABP content ranged from 2-10 ng/mg cytosolic protein, while the L-FABP content was between 0.1-0.8 ng/mg cytosolic protein (data not shown).

Table 3.3 : *Distribution of H-FABP in cardiomyocytes and endothelial cells of adult rat heart as found by immuno-electron microscopy studies. Results were obtained by semiquantitative analysis of electronmicrographs from adult rat heart tissue, as described in Materials and Methods, and are given as means ± SD for the indicated number of rat heart preparations. %, values are relative to the cardiomyocytal cytoplasm (100%).*

Cell-type	Compartment	Dots/μm^2	%
Cardiomyocyte	cytoplasm	327 ± 27 (3)	100
	nucleus	0.5 ± 0.1 (3)	0.2
	mitochondria	0.9 ± 0.8 (4)	0.3
Endothelium	cytoplasm	2.1 ± 0.7 (4)	0.6

Immunohistochemical detection of FABP

Fig 3.3 shows a representative example of an electron micrograph of H-FABP staining of rat heart tissue. Gold particles were counted in different cell-types and cell-organelles as dots per square μm in preparations obtained from four animals. The results of these semiquantitative measurements are shown in table 3.3. In endothelial cells the number of dots per square μm is 0.6% of that of the cytoplasm of cardiomyocytes. Electron microscopy of rat heart tissue using antibodies raised against L-FABP showed no specific immunostaining.

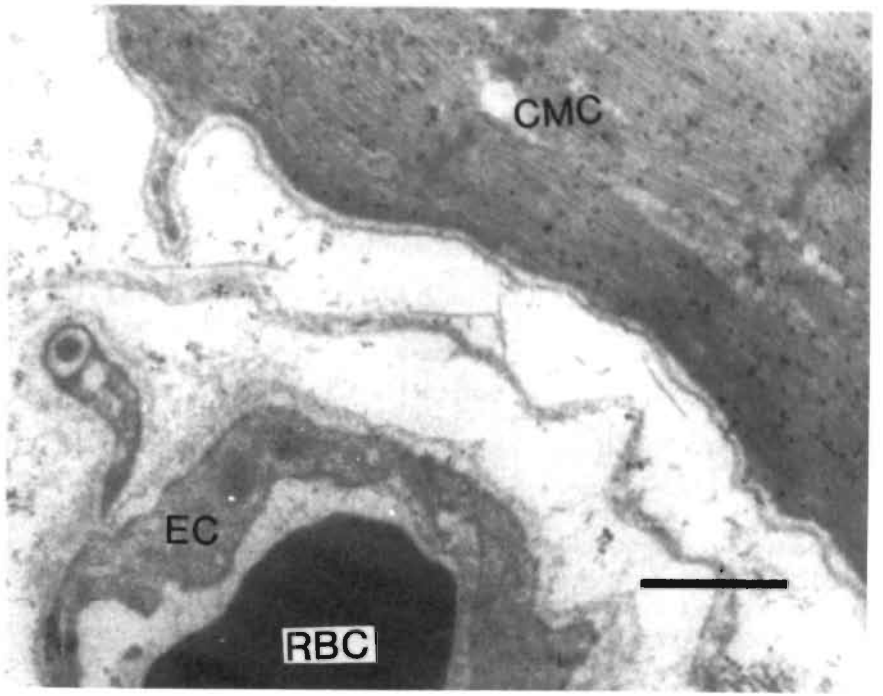

Figure 3.3 : *Immuno-electron microscopy of rat heart tissue using affinity purified antibodies against H-FABP. CMC : cardiomyocytal cytoplasm, EC : endothelial cell, RBC : red blood cell. Bar represents 1 μm*

Assay for FA-binding activity

To verify the assay, a mixture of purified rat H-FABP and [^{3}H]-palmitic acid was injected onto the column. A single protein peak at about 15 kDa was found by absorbance (280 nm), and a corresponding peak of radioactivity by measurement with the radio-chromatography detector. Similar results were found with recombinant rat L-FABP and I-FABPs. Thereafter, FA coelution was measured in 105,000 g supernatants of rat heart and liver and of cardiomyocyte and endothelial cell homogenates (fig 3.4). In the heart and cardiomyocyte homogenates myoglobin (measured by absorbance) eluted before FABP (radioactivity peak), which is in agreement with the larger molecular size of myoglobin (18 kDa) in comparison to FABP (15 kDa). Dilution series of the heart

homogenate showed the detection limit of this HPGPC coelution technique to be on the order of 0.5 μg of FABP. The endothelial cell homogenate, however, did not show any FA coelution, even when about 800 μg of endothelial cytosolic protein was injected onto the column. Hence, assuming the presence of an unknown FABP with comparable affinity for FA as H-FABP, the endothelial cell FABP content would not exceed 0.6 mg/g cytosolic protein.

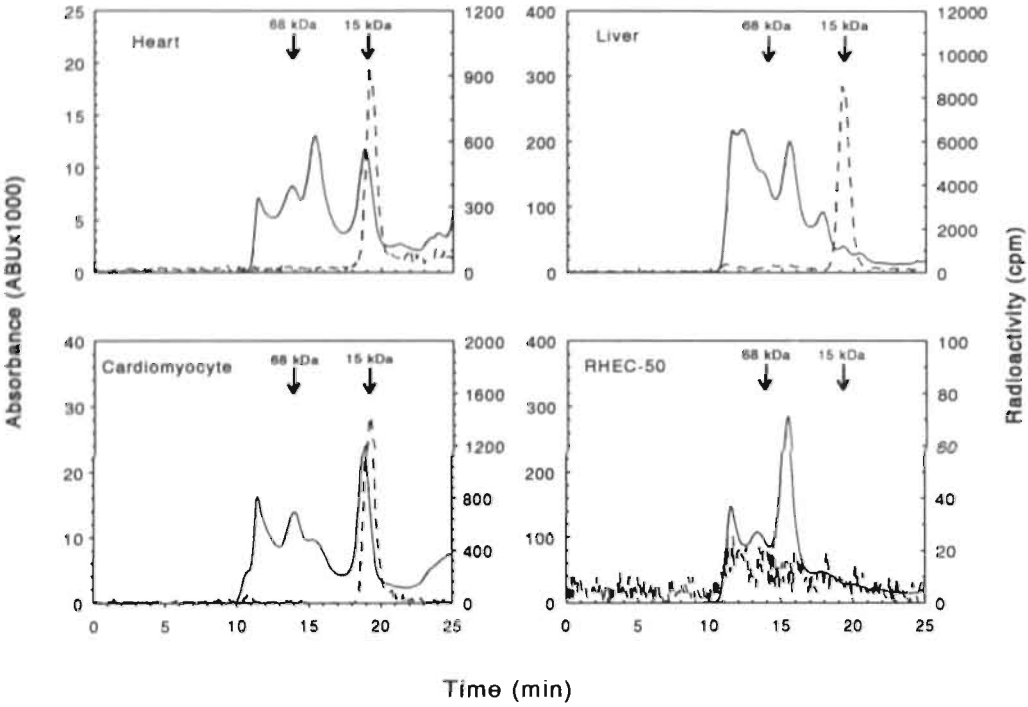

Figure 3.4 : *High performance gel permeation chromatography (HPGPC) of homogenates from rat heart (88 μg), liver (430 μg), cardiomyocytes (100 μg) and RHEC-50 (700 μg) in the presence of [^{3}H-] palmitate. Bold lines indicate absorbance and dashed lines the radioactivity as measured by radiochromatography detection. 68 kDa marks the elution position of bovine serum albumin and 15 kDa that of purified FABP.*

To increase the resolving power of HPGPC analysis of endothelial cells, data of seven HPGPC runs were compiled, so as to improve the signal to noise ratio (fig 3.5). As shown in fig 3.5, signals of FA coelution could be demonstrated. The first radioactivity peak eluted in the void volume (ca. 11 min) and the second radioactivity peak at about 16 min, the latter representing proteins with a molecular mass of about 40 kDa. However, no detectable radioactive signal was observed in the 15-kDa region (fig 3.5).

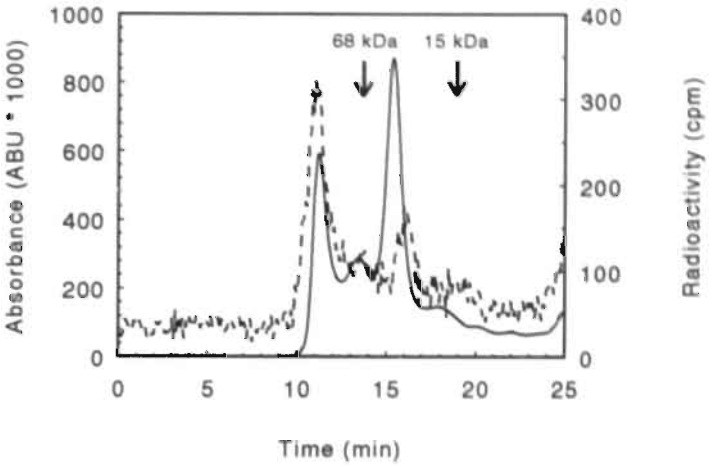

Figure 3.5 : *Compilation of 7 runs of high performance gel permeation chromatography (HPGPC) with coelution of [^{3}H-] palmitate of homogenates from rat heart endothelial cells. Bold line indicates absorbance and the dashed line the radioactivity as measured by radiochromatography detection. 68 kDa marks the elution position of bovine serum albumin and 15 kDa that of purified FABP.*

DISCUSSION

FA are the main energy source of the heart under physiological conditions (5,34). The manner by which these FA are transported from the blood compartment across the capillary wall to the interstitial space is incompletely understood. Because FA are poorly soluble in an aqueous environment, a variety of hypotheses have been presented to explain the experimentally observed flux of FA (3). A likely hypothesis is that FABP can raise the solubility of the FA and thereby their diffusional flux through the endothelial cytoplasm (9,32). The presence of H-FABP in the endothelial cells has been reported by several authors, but the endothelial content of H-FABP differs considerably among these various investigations. Fournier et al. (9) were the first to describe the presence of H-FABP in rat heart endothelial cells. With an immunohistochemical technique using anti-H-FABP antiserum and subsequent labelling with protein A-gold, they estimated the endothelial H-FABP content to be 3.3 mg per g of wet tissue. With an endothelial cell volume of about 0.4 pl/cell (2) and a cytosolic protein content of 50 pg/cell (17,28), the H-FABP content would be about 26 mg/g cytosolic protein. Paulussen et al. (22), using an ELISA of the antibody capture type, found the H-FABP content of isolated endothelial cells of either rat heart or human umbilical vein to be about 8 mg/g cytosolic protein. Linssen et al. (18), however, using the same ELISA technique, found the H-FABP content of cultured endothelial cells from rat heart to be 8 μg/g protein which is about 3 orders of magnitude lower than the values reported earlier. Finally, Robers et al. (23) reported that the H-FABP content of bovine aortic endothelial cells amounted to 90 μg/g protein. In the present study a more systematic approach was followed to identify and quantify

FABP types in endothelial cells from rat heart, making use of a variety of biochemical and molecular biological techniques.

FABP in endothelial cells from rat heart

Since H- and L-FABP are the sole FABP-types known to occur in more than one type of tissue (e.g. I-FABP is confined to intestinal enterocytes), we focused on these two members of the FABP-family. In addition, attempts were made to demonstrate the presence of FA-binding activity in cytosolic preparations from endothelial cells to functionally identify those proteins which could play a role in FA transport in the endothelial cell cytoplasm.

By northern blot analysis H-FABP and L-FABP mRNA could not be detected in endothelial cells, which indicates that no substantial amounts of mRNA of these proteins are present in the cells. However, with RT-PCR, mRNA of both H- and L-FABP were detected in the endothelial cells. Theoretically, the presence of a single target molecule is sufficient for a positive result in PCR. The fact that RT-PCR identified these two FABP mRNAs and that northern blot analysis showed no bands demonstrates that mRNA concentrations of H-FABP and L-FABP are very low. In addition to H-FABP and L-FABP mRNAs, as found by PCR in heart and liver, respectively, we also found a signal for H-FABP in liver and L-FABP in heart tissue. The presence of H-FABP mRNA in liver tissue can be explained by smooth muscle cells, containing the heart type of FABP (15), in hepatic blood vessel walls. The L-FABP signal in PCR of total heart tissue will likely result from the low amount of mRNA of L-FABP in endothelial cells.

Using specific and sensitive sandwich ELISAs, the amounts of H-FABP and L-FABP in endothelial cells were measured at the protein level. Quantities monitored were at least 3 orders of magnitude lower than detected with the same assay in heart and liver tissue, respectively. The H-FABP concentrations found in the endothelial cells are in accordance with values earlier reported by Linssen et al. (18), but appreciably lower than reported by others (9,22,23). This discrepancy may relate to the use by these latter investigators of either endothelial cell cultures containing some cardiomyocytes or less specific assay methods (e.g. direct ELISA and/or full antisera).

Immuno-electron microscopy was performed on fixed rat cardiac tissue to estimate the H-FABP concentration in endothelial cells in their original biological matrix. These studies showed that the concentration of H-FABP in endothelial cells is less than 1% of that in cardiomyocytes. The results corroborate well with the molecular biological and immunochemical studies in the cultured endothelial cells (see above).

An assay for FA-binding activity used to study the presence of other types of FABP in the endothelial cells revealed no FA-binding in the 15-kDa region, which is the molecular size of all known FABPs (10). Taking into account the detection limit of the assay, this finding indicates that the concentration of a cytoplasmic FABP with comparable FA affinity in the endothelial cells would be lower than 0.6 mg/g cytosolic protein, i.e. less then 4% of the amount of the FABP in heart or liver tissue. When the results of all experiments were compiled, minor FA appeared to coelute in the void volume . This might be explained by the ability of unbound FA to form micelles or other aggregates. Such large structures would elute in the void volume. Some FA coelution was observed at about 16 min, corresponding with a molecular mass of ca. 40 kDa, which is possibly the result of a FA-binding protein of this molecular mass. Indeed, in rat endothelial cell a membrane-bound 40 kDa FABP has been found (38), that could be partly solubilized by our homogenization procedure. An alternative explanation for the observed FA binding

in this region may be aspecific (low-affinity) binding of FA to a number of proteins with a molecular mass of ca. 40 kDa, which would then cause a higher background radioactivity in the areas were these proteins elute. We cannot exclude, however, that there is a cytoplasmic 40 kDa protein capable of binding FA. The low signal indicates that either minor amounts of this protein are present or the affinity of this protein for palmitate is very low. In both cases a physiological role for such a 40 kDa protein in trafficking FA through the endothelial cytoplasm seems unlikely.

Mechanisms of FA transport across endothelial cells

Our data indicate that rat heart endothelial cells contain only minor amounts of cytoplasmic FABP, suggesting that FA transport over the cytoplasm of these cells can be mediated by FABP only to a low extent. The rate of uptake of FA in rat heart, under normal physiological conditions, is about 100 nmol/min per gram wet weight of tissue (4,8,19). Tschubar et al. (32) calculated the required concentration differences of FA over the endothelial cytoplasm to explain the FA flux by diffusion in two hypothetical situations, i.e. (i) assuming no FABP present, so that the observed FA uptake is to be explained only by aqueous diffusion of 'free' (non-protein bound) FA, and (ii) assuming FABP is present in a concentration of ca. 26 mg/g cytosolic protein as published by Fournier et al. (9). Their calculations reveal that FA uptake-rates of up to 100 nmol/min per g wet weight of tissue can be explained by aqueous diffusion of 'free' FA, while in the presence of FABP larger FA uptake-rates are feasible. Nevertheless, on the basis of the supposed abundance of FABP these investigators concluded that transport of FA over the endothelial cytoplasm would take place almost exclusively by diffusion of FA bound to FABP (32). In view of our present data, however, this conclusion is questionable. This is illustrated by a calculation of the contribution of the diffusional flux of FABP-bound FA to the total FA-flux across the endothelial cytoplasm, using FABP-concentrations as measured with our immunochemical assays.

For this calculation we assume separate uni-directional fluxes of FABP complexed and non-protein bound FA from the luminal to the abluminal membrane of the endothelial cell, similar to calculations made for cardiomyocytes (37). This diffusion can be described by Fick's law :

$$Q/t = D \cdot A \cdot \Delta C/u.$$

The total flux of FABP-FA and unbound FA can then be described as

$$Q/t = (Q/t)_{FABP\text{-}FA} + (Q/t)_{FA}$$

$$Q/t = D_{FABP} \cdot A \cdot \Delta C_{FABP\text{-}FA}/u + D_{FA} \cdot A \cdot \Delta C_{FA}/u.$$

In this formula **Q/t** is the migration of solute per unit of time, or diffusional flux (mol/s), **D** is the diffusion coefficient, $D_{FA} = 6.5 * 10^{-6}$ cm^2/s (24) and $D_{FABP} = 1 * 10^{-7}$ cm^2/s (37) and **A** is the surface through which diffusion takes place. This latter surface can be calculated using the measurements of Anversa et al. (2) and taken into account the differences in surface area at the luminal and abluminal sides of the capillary wall (14,37). For 1 g of rat heart tissue the average diffusion surface **A** was found to be ca. 795 cm^2. **ΔC** is the concentration difference in mol/cm^3 and **u** represents the distance

of FA transport, i.e. the distance between the luminal and abluminal membranes of the endothelium, which is approximately $3*10^{-5}$ cm (3,32). Substituting these values the diffusional flux can be calculated as follows:

$$Q/t = (Q/t)_{FABP\text{-}FA} + (Q/t)_{FA}$$

$$Q/t = 2.7 \cdot \Delta C_{FABP\text{-}FA} + 172 \cdot \Delta C_{FA}$$

The total content of (H- and L-) FABP in the endothelial cell is ca. 6 μg/g of cytosolic protein (table 3.2). With an endothelial cell volume of about 0.4 pl/cell (2) and a cytosolic protein content of 50 pg/cell (17,28), this corresponds to a FABP concentration in the endothelial cytoplasmic space of ca. 50 nM = 50 pmol/cm^3. The maximal flux of FABP complexed FA takes place if at the luminal side 100% and at the abluminal side none of the FABP were complexed with FA. Hence, the maximal value of $(Q/t)_{FABP\text{-}FA}$ amounts to $2.7 \cdot 50 = 135$ pmol/s = 8 nmol/min. The latter value cannot explain the experimentally observed total FA-flux of ca. 100 nmol/min. Considering the second term of the above-mentioned equation, however, calculation shows that the flux of non-protein bound FA can reach a value of 100 nmol/min (1.7 nmol/s) provided that the concentration gradient of FA over the endothelial cytoplasm amounts to 10 pmol/cm^3 = 10 nM. Such gradient is in reasonable agreement with the calculations of Tschubar et al. (32), and within the range of physiological FA concentrations. Taken together, we propose that the main mechanism by which FA are transferred from the luminal to the abluminal membrane through the endothelial cell cytoplasm is aqueous diffusion of non-protein bound FA.

It should be kept in mind that still other mechanisms may also contribute to FA transfer across the endothelial cell barrier. For instance, receptor mediated transcytosis of albumin-FA complexes across cultured endothelial cells has been reported (1), but this process is most likely too slow to permit a major contribution to the total rate of FA-uptake by the heart (3,34). An alternative mechanism is the transfer of FA through the plasmalemmal bilayer as proposed by Scow et al. (27). Calculations performed by Bassingthwaighte et al. (3) makes this possible mechanism less likely, but a minor contribution to the total FA-flux can not be excluded.

In summary, molecular biological, immunochemical and functional (FA-binding) assays revealed conclusively that the endothelial cells of rat heart contain only minor amounts of cytoplasmic FABP. Therefore, FA transfer across the endothelial cytoplasm will most likely take place predominantly by aqueous diffusion of non-protein bound FA.

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CHAPTER 4

PUTATIVE MEMBRANE FATTY ACID TRANSLOCASE AND CYTOPLASMIC FATTY ACID-BINDING PROTEIN ARE CO-EXPRESSED IN RAT HEART AND SKELETAL MUSCLES

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ABSTRACT

A membrane protein (FAT) homologous to CD36 has recently been implicated in the binding and transport of long-chain fatty acids (FA). Expression of this protein in rat heart, skeletal muscles and in isolated cardiac cells was studied. Changes in expression during development of the heart were also examined. Expression of FAT was compared to that of the cytoplasmic fatty acid-binding protein (H-FABP) to determine whether co-expression, indicative of related biological functions, could be demonstrated.

FAT and H-FABP mRNAs showed a similar muscle tissue distribution and similar cellular localization in the heart. During development, heart mRNA levels for both proteins were upregulated in the same way.

In conclusion, expression of FAT and H-FABP in muscle tissues and cell-types with high FA metabolism and the upregulation of mRNA levels associated with heart development, when FA utilization increases, support the suggested role of both proteins in FA metabolism.

INTRODUCTION

Long chain fatty acids (FA) are important substrates for energy production in both heart and skeletal muscles (23,24). In heart, FA are the preferred substrate under physiological conditions. In skeletal muscles, FA contribution to total ATP formation varies with the type of muscle as well as with intensity and duration of exercise (24). For example, the soleus muscle with its high proportion of slow-twitch oxidative fibers is more dependent on FA metabolism than the extensor digitorum longus muscle (EDL), a predominantly glycolytic muscle with a low proportion of these fibers (22,26). FA in the circulation and in the interstitial fluid are bound to albumin (20). After transfer of the FA moiety from the vascular compartment to the interstitial space, FA are rapidly taken up by muscle cells and transported to mitochondria for subsequent oxidation.

A variety of proteins in the plasma membrane and in the cytoplasm have been shown to function as receptors for FA and were hypothesized to be involved in the transport of FA from the extracellular fluid to oxidation or esterification sites. Inside the myocyte, a 15 kDa protein (Heart-type or H-FABP), member of a large family of small FA-binding proteins, is abundantly present and has been linked to the intracellular transport of FA (5,27). At the level of the plasma membrane, a 40 kDa membrane-bound fatty acid-binding protein, identified on both endothelial and muscle cells, was postulated to mediate FA uptake by these cells (19,28). Recently, a 88 kDa membrane protein was identified in rat adipocytes by labelling with sulfo-*N*-succinimidyl derivates of FA under conditions where FA uptake was significantly inhibited (9,10). This protein, termed fatty acid translocase (FAT) for its postulated role in FA transport, was highly expressed in heart and skeletal muscles (1) and, presumably, would contribute to sequestration and uptake of FA by these tissues. FAT was found to be highly homologeous to CD36, a membrane protein found in monocytes, platelets and endothelial cells (7).

We hypothesized that if both FAT and H-FABP are involved in the metabolism of FA, they should be expressed in the same tissues and cell types. Furthermore, expression should exhibit similar regulation during peri- and postnatal development when FA metabolism increases in the heart (6).

The aim of the study was to examine the expression of FAT and H-FABP in rat heart, in skeletal muscles with predominantly oxidative or glycolytic metabolism and in cell types isolated from heart tissue. Development-related changes in expression during heart maturation were also investigated. In addition, H-FABP and FAT expression were studied in control and diabetic rat heart and skeletal muscles. The data indicate co-expression of FAT and H-FABP in muscle consistent with their postulated functions in FA transport.

MATERIALS AND METHODS

Tissues and cells

Heart, liver, adipose tissue, soleus muscle and extensor digitorum longus muscle (EDL) were dissected from adult (10 weeks old) Wistar Kyoto (WKY) rats. Rat hearts were also obtained at birth (day 0) and at 2, 6 and 21 days. To obtain fetal hearts, pregnant rats were sacrificed at day 21 of pregnancy (day -1). All tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

Cardiomyocytes were isolated from adult rat heart as described by Linssen et al. (13,15). Isolation of neonatal cardiomyocytes was performed with neonates of 0-3 days of age as previously described (12,18). The isolated cardiomyocytes were harvested one day after isolation. Two lines of endothelial cells (RHEC-50 and RHEC-116) and fibroblast-like cells, all derived from rat heart, were also used (13-15).

Experimental diabetes

Six male WKY rats of ca. 250 g were rendered diabetic by a single injection of 17.5 mg streptozotocin in 1 ml citrate buffer (70 mg/kg body weight). Six control animals received an injection with citrate buffer alone. Three weeks later the animals were sacrificed. At this time the mean blood glucose concentration of the streptozotocin injected rats was 22 ± 3 mmol/l, while control rats had a glucose level of 7.0 ± 0.7 mmol/l (means ± SD for six animals). From each animal, the heart, soleus and EDL muscles and the liver was quickly removed and frozen in liquid nitrogen for subsequent RNA isolation and for tissue homogenization.

Northern and slot blotting

Diethyl pyrocarbonate (DEPC) treated water (17) was used for all steps involved in RNA isolation (2). For Northern blotting, electrophoresis of RNA (7 μg) was followed by transfer to a nylon membrane (Hybond-N, 0.45 micron, Amersham, Little Chalfont, UK) by capillary action in 10 x saline sodium citrate buffer (10 x SSC; 1.5 M NaCl, 0.17 M $Na_3C_6H_5O_7$, pH 7.0). Slot blotting of RNA (0.2, 1 and 5 μg) onto a nylon membrane (Hybond N^+, Amersham) was carried out using a 48 slot (0.75 x 7.5 mm) convertible manifold filtration system (Gibco, Life Technologies, Gaithersburg, MD, USA). RNA was fixed to membranes by heating at 80°C for 15 min followed by crosslinking under ultraviolet light (0.4 J/cm^2). To ensure that RNA was intact and evenly loaded and to check transfer to the nylon membrane, RNA was stained with methylene blue to visualize 28S and 18S ribosomal bands.

RNA was probed with the cDNAs for FAT (1) and H-FABP (11) labelled using a rediprime kit from Amersham. Filters were prehybridized (one hour) and hybridized (overnight) at 58°C in 6 x SSC containing 0.5 g/l each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, 0.5% SDS and 100 μg/ml salmon sperm DNA. To remove non-

specific binding, filters were washed for periods of 30 min, first at 42°C with 2 x SSC followed by 1 x SSC containing 0.1% SDS, then at 55°C with 0.2 x SSC containing 0.1% SDS and finally with 0.1 x SSC containing 0.1% SDS. Filters were exposed to X-ray film at -80°C and to imaging screens for scanning and quantitation (Phosphor Imager, ImageQuant; Molecular Dynamics, Sunnyvale, CA, USA).

Sandwich ELISA for H-FABP
All steps were performed on ice or at 4°C. Tissues were cut and homogenized (1.5-10% (w/v), depending on the amount of tissue available) in SET-buffer (10 mM Tris, 2 mM EDTA, 0.25 M sucrose, pH 7.4) using an Ultra-Turrax homogenizer (IKA Werke, Breisgau, Germany). The homogenates then were sonicated 4 times for 15 s. Cultured cells were harvested using trypsin/EDTA treatment, or using a rubber policeman in phosphate buffered saline (PBS) and sonicated 4 times for 15 s. Cell and tissue homogenates were stored at -80°C until use. Protein concentration was measured according to Lowry et al. (16) using bovine serum albumin as standard. Rat H-FABP was measured using an indirect non-competitive sandwich ELISA as described by Vork et al. (26).

RESULTS AND DISCUSSION

The expression of FAT and H-FABP was studied in rat heart, soleus and extensor digitorum longus (EDL) muscle to examine whether both proteins show similar distribution among these tissues. In addition, expression of both proteins was studied during heart development and streptozotocin-induced diabetes. Co-expression of FAT and H-FABP under these conditions would indicate related biological functions.

Tissue distribution of FAT and H-FABP.
Northern blot analysis of RNA from rat heart, soleus and EDL muscles and from cell types isolated from rat heart is shown in fig 4.1. Adipose and liver RNA were included as controls. As shown in fig 4.1 (lanes 3-8) both FAT and H-FABP probes hybridized with RNA from skeletal muscles, from adult and fetal heart and from cardiomyocytes obtained from adult or neonatal rats. Single bands were detected at about 2.9 kb for FAT (1) and 0.75 kb for H-FABP (11). The expression level of FAT, as assessed by phosphor imager quantitation, and given relative to that in heart (set at 100%), was in soleus 72% and 54% and in EDL 32% and 14% (two animals). Similarly, the expression level of H-FABP was in soleus 43% and 33% and in EDL 12% and 5% of that of heart (two animals). The latter data are in close agreement with H-FABP protein levels measured in heart, soleus and EDL (i.e. 5.7 ± 1.1, 2.6 ± 0.2 and 0.5 ± 0.1 mg H-FABP/g protein, respectively, table 4.1). Consistent with previously published data (1,11), no signal was detected for either FAT or H-FABP in liver RNA while the FAT probe, but not the H-FABP probe, hybridized with RNA from adipose tissue. No hybridization was found by either FAT or H-FABP probes with RNA from endothelial (RHEC) or fibroblast-like cells derived from rat heart. For H-FABP, the data are consistent with the very low levels of H-FABP protein previously reported in liver tissue and in heart endothelial and fibroblast-like cells (15,25).

The findings with FAT support its co-expression with H-FABP in muscle cells and tissues. FAT expression was higher in the soleus compared to the EDL muscle. This suggests, as for H-FABP, that FAT expression is linked to oxidative rather than to

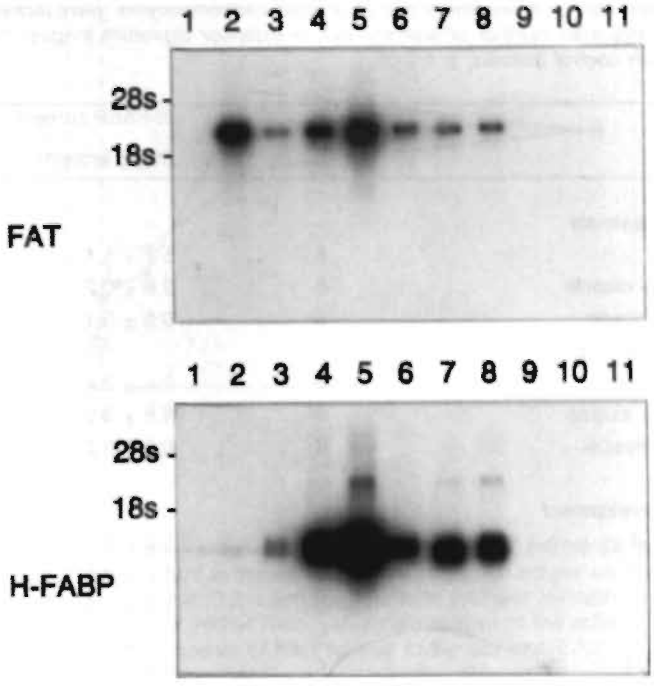

Figure 4.1 : *Northern blot analysis of total RNA from rat tissues and isolated cells of rat heart probed with FAT and H-FABP cDNA. The results of a representative experiment are shown. Lanes: 1, liver; 2, adipose tissue; 3, extensor digitorum longus (EDL) muscle; 4, soleus muscle; 5, adult heart; 6, fetal heart; 7, adult cardiomyocytes; 8, neonatal cardiomyocytes; 9, RHEC-50; 10, RHEC-116; 11, cardiac fibroblast-like cells.*

glycolytic metabolism (22,26). Expression was prominent in the adult heart, a tissue highly dependent on FA utilization for energy conversion under normal conditions (23). The lower expression of both proteins observed in fetal heart is consistent with the association of increased FA metabolism with heart development (6). In isolated cardiomyocytes from both neonatal and adult hearts, FAT and H-FABP expression was lower than in the adult heart tissue and approximated that in fetal heart. This finding suggests that isolation and short-term culturing of adult cardiomyocytes results in marked decreases in mRNA levels for both proteins.

FAT and H-FABP expression during cardiac development

The higher level of FAT mRNA observed in adult versus fetal heart suggests that expression of FAT, like that of H-FABP (3,11), is upregulated during heart development as FA utilization by the tissue increases (6,23). To examine this in more detail, RNA samples obtained from rat hearts at different stages of development (from prenatal day -1 to postnatal day 70) were probed with the cDNAs for FAT and H-FABP. Liver RNA was used as a negative control. As shown in fig 4.2, expression of FAT in the heart followed a pattern similar to that of H-FABP and increased markedly (5-fold) with age,

Table 4.1 : *H-FABP contents of heart and skeletal muscles from adult control and diabetic rats, of rat heart during development and of isolated cardiomyocytes. Data represent means ± SD for the indicated number of animals. EDL = extensor digitorum longus. * Statistically significant from control animals, $p < 0.05$.*

Tissue	n	H-FABP content (mg/g protein)
Control animals		
heart	6	5.7 ± 1.1
soleus muscle	6	2.6 ± 0.2
EDL muscle	6	0.5 ± 0.1
Diabetic animals		
heart	6	7.4 ± 2.4
soleus muscle	6	4.8 ± 0.8 *
EDL muscle	6	0.8 ± 0.2 *
Heart development		
- day -1	5	1.2 ± 0.1
- day 0	5	1.2 ± 0.1
- day 2	5	1.7 ± 0.1
- day 6	5	2.2 ± 0.2
- day 21	5	4.1 ± 0.3
- day 70	5	5.4 ± 0.8
Isolated cardiomyocytes		
- neonatal	2	2.7 ; 1.8
- adult	7	3.9 ± 1.6

reaching mature levels around postnatal day 21. For H-FABP, the changes in mRNA levels were always paralleled by changes in H-FABP protein content (table 4.1). It would be of interest to study whether this also applies to FAT but this will have to await the availability of antibodies for this protein.

Expression of FAT and H-FABP during diabetes

Some preliminary studies were performed to investigate the possible changes in FAT and H-FABP expression in rat heart and skeletal muscles during streptozotocin-induced diabetes, since diabetes is known to shift cardiac energy metabolism from carbohydrate to fatty acid utilization (4). RNA samples from one successful isolation of RNA from heart as well as from the skeletal muscles, were used in northern blot analysis. Liver RNA served as negative control. This northern blot was probed with FAT and H-FABP cDNA and is shown in fig 4.3. FAT mRNA levels tend to be higher in heart and skeletal muscles of the diabetic animal as compared to the control rat. The level of H-FABP mRNA does not differ much between the hearts of the control and diabetic animal. In soleus and EDL

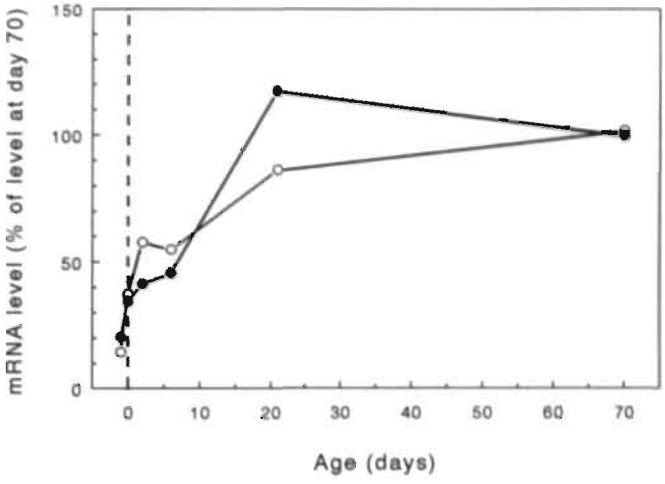

Figure 4.2 : *Semiquantitative assessment of the levels of FAT mRNA (o) and of H-FABP mRNA (●) during development in the rat. Counts on phosphor imager were quantitated for three different amounts of RNA (0.2, 1 and 5 μg) for both FAT and H-FABP. In each case the counts on day 70 were set at 100%. Thus, values are relative to the mRNA level at day 70 and are average for three amounts of RNA applied to the slot blot.*

muscle, however, H-FABP signals appear to be stronger in the diabetic animal as compared to control animal (see fig 4.3).

In a separate experiment, RNA samples from the hearts of the six diabetic and the six control animals were used for northern blot analysis and mRNA levels were subsequently quantified as described in the methods section. H-FABP mRNA levels were increased 28 ± 36 % in the diabetic animals relative to control animals. The mean FAT mRNA level in diabetic rat heart was 67 ± 56 % higher than that in control hearts. However, both the H-FABP and FAT increases are not statistically significant, which may be due to limitations of the used method to accurately measure these relatively small differences.

H-FABP protein levels in diabetic heart and skeletal muscles were assessed using the sandwich ELISA and the results are shown in table 4.1. The increase in H-FABP in diabetic heart compared to control heart is 30%, but due to the large scatter this increase did not reach the level of significance. The tendency to increase is, however, in close agreement with the northern blot results and with earlier studies on H-FABP expression in streptozotocin-induced diabetic rat heart (4). On the other hand, the increase in H-FABP expression in the skeletal muscles soleus and EDL of diabetic animals is statistically significant. To delineate the biological relevance of the differences found in FAT and H-FABP expression in diabetic animals more detailed studies have to be performed. Interestingly, Greenwalt et al. (8) reported recently that CD36 (which is probably identical to FAT, see chapter 2) is upregulated in heart in murine models of diabetes.

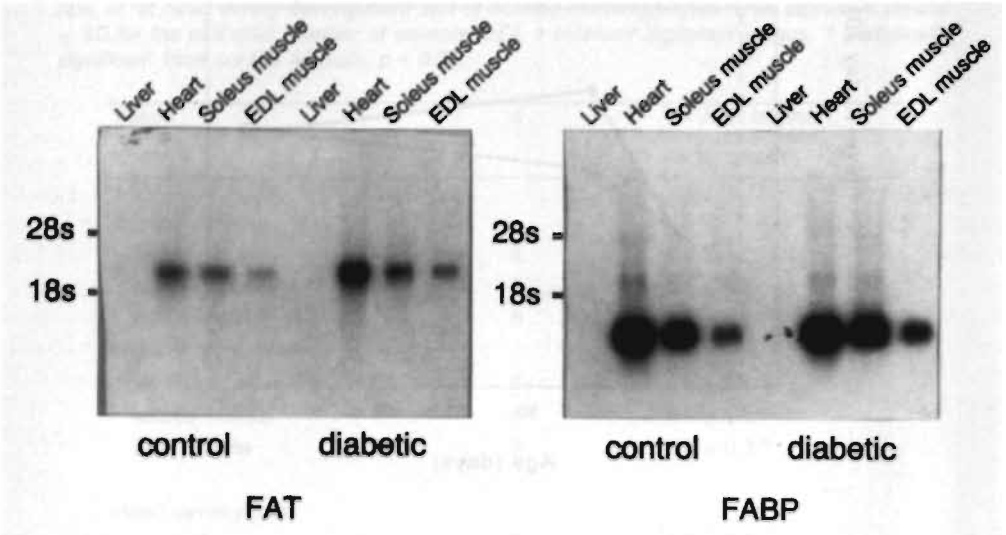

Figure 4.3 : *Northern blot analysis of total RNA from liver, heart, soleus and extensor digitorum longus (EDL) muscle from a control and a streptozotocin-induced diabetic rat probed with FAT and H-FABP cDNA.*

Concluding remarks

The present study demonstrated a similar distribution of FAT and H-FABP mRNAs in heart and skeletal muscle tissues and in several cell types isolated from the heart. A similar upregulation of the mRNAs of both proteins was observed in the heart during development. In addition, preliminary results suggest that FAT expression might be increased in heart and skeletal muscles during diabetes.

When our studies had been finished, Spitsberg and colleagues reported an association and co-expression of H-FABP and CD36 in the bovine mammary gland (21). Complexes of CD36 and H-FABP, most likely formed by binding of H-FABP to the cytoplasmic segment of CD36, were found in milk fat globule membranes (21). These findings are in correspondence with the co-expression we found in rat muscles. The association found between CD36 and H-FABP (21) is also very important for the mechanism of FA uptake across the plasmamembrane (see also fig 2.3).

In general, expression of both FAT and H-FABP appeared to correlate with the oxidative capacity of the tissue. The data are consistent with the postulated roles of FAT and H-FABP in, respectively, membrane and cytoplasmic transport of fatty acids.

ACKNOWLEDGEMENT

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CHAPTER 5

STABLE TRANSFECTION OF PUTATIVE FATTY ACID TRANSLOCASE (FAT) IN A RAT HEART MUSCLE CELL-LINE (H9c2)

INTRODUCTION

The precise mechanism of the transport of long chain fatty acids (FA) across the sarcolemma of cardiomyocytes is a matter of considerable debate (see chapter 2). Some investigators favour passive diffusion of FA through the phospholipid bilayer surrounding the cardiac cell as the main transfer process (11), while others reported data suggesting that the sarcolemmal transport of FA is mediated by membrane associated proteins (13,14). One of the proteins hypothesized to be involved in trans-sarcolemmal FA transport is fatty acid translocase (FAT) (1,15). This protein was identified in adipocyte membranes by covalent labelling with FA derivatives, upon which FA uptake was inhibited by about 70% (5). In chapter 4, it was demonstrated that FAT shows a co-expression with H-FABP in rat heart and skeletal muscles, indicating related functions in these tissues. Although these studies suggest that FAT is involved in the cellular uptake of FA (1,3-5,15), the precise role of this protein in this uptake process is still unclear. FAT could act as a translocator of FA, or it could trap the FA and create a steeper transmembrane gradient. In addition there could be interactions between FAT and extracellular or intracellular FA-binding proteins (see fig 2.3).

To study the role of FAT in FA-uptake in more detail, we performed a stable transfection of FAT in the clonal rat muscle cell-line H9c2. This cell-line is derived from embryonic rat heart tissue and initially was found to have properties of skeletal muscle cells (8). The cells propagate as mononucleated myoblasts and upon reaching confluency they can form multinucleated tubular structures (8). However, subsequent studies by other investigators showed that H9c2 also exhibit specific properties of cardiomyocytes, such as the expression of the cardiac isoform of L-type calcium channel (7,9,12) and the tissue-specific splicing protein SmN (2). It is concluded that this cell-line has both cardiac and skeletal muscle characteristics (9). As H9c2 cells normally do not express FAT as found by northern blot analysis (unpublished observations), we reasoned that this cell-line forms a suitable model system to study FAT function.

The objective of the present study is to investigate FA-uptake in control and FAT transfected H9c2 cells. It is hypothesized that FA uptake is correlated with FAT expression and therefore will be increased in H9c2 cells upon transfection with this protein. This study, however, was not completed at the time of writing this thesis. In this chapter, the results of the transfection are presented and the use of the obtained model system in future investigations will be discussed.

MATERIALS AND METHODS

Cell culture

H9c2(2-1) cells were obtained from the American Type Culture Collection (ATCC, CRL1446) and were used between passage 20 to 30. These cells, which will be designated H9c2 throughout this thesis, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 9% (vol/vol) fetal calf serum (FCS, SEBAK, Aidenbach, Germany) and 0.05% gentamycin, which will further be called standard medium. Cells were plated at a density of about 10^4 cells/cm^2 in a 100 mm dish (Falcon, Becton Dickinson, Plymouth, UK) and were grown under 5% CO_2 in water saturated air.

Transfection
Three 100 mm Falcon dishes of 50% confluent H9c2 cells (passage 23) were transfected with a mixture of the plasmids PSG5-FAT (10 μg) and PMAMneo (0.5 μg). PSG5-FAT was a gift of dr. P.Grimaldi from the University of Nice, France, and was constructed by inserting the coding region of FAT (about 1.4 kb) into the BamH1 site of the eukaryotic expression vector PSG5 (Stratagene, La Jolla, CA, USA). The FAT sequence is under the control of the early SV40 promotor and is preceded by intron II of the rabbit ß-globin gene, which facilitates splicing. The plasmid also contains a polyadenylation signal, which should increase the expression level of the FAT in the transfected cells. pMAMneo (ClonTech, Palo Alto, CA, USA) was included in the transfection for selection purposes and contains the selectable marker gene 'neo' under the control of the SV40 promotor. Cotransfection of both plasmids into H9c2 was performed using the lipocarrier DOTAP (Boehringer, Mannheim, Germany), exactly according to the manufacturers instructions. The transfection mix was added to the cells at the end of the day and the transfection was carried out overnight (incubation time, approximately 15 hrs).

Selection of stably transfected cells
After overnight incubation, the transfection mix was removed and fresh standard medium was added to the cells. At the end of the same day the cells were trypsinized (0.25% trypsin from Gibco, in PBS), replated in three different dilutions (2x, 4x and 8x) and selection medium was added to the cells. Selection medium consisted of standard medium supplemented with 400 mg/l geneticin (G418, Gibco). Untransfected H9c2 cells (control) were treated identical, so as to check the efficiency of the selection. Selection was carried out for 4 weeks and control cells all died within 3 weeks. On all transfected dishes, cells were growing as colonies, and several colonies were selected and isolated using small squares of Whatmann 3 mm paper soaked in 0.25% trypsin buffer. Twenty-six colonies were isolated, of which 15 could be maintained as cell-lines. These cell-lines were propagated until at least 10 aliquots of each cell-line had been stored in liquid nitrogen. After the selection, cells were cultured in maintenance medium, which consisted of standard medium supplemented with 200 mg/l geneticin.

Northern blotting
To check mRNA levels of FAT in the cell-lines obtained after selection, 5 μg of total RNA (isolated using Trizol reagens from Gibco) was separated by electrophoresis, and blotted on a nylon membrane (Hybond-N, 0.45 micron, Amersham, Little Chalfont, UK) as described earlier (15). RNA was fixed by drying for 15 min at 80°C followed by crosslinking under ultraviolet light (0.4 J/cm^2). Subsequently, RNA was stained with methylene blue and the 28S and 18S ribosomal bands were quantified using a hand scanner (Primax, Zeist, the Netherlands) and the software programs FinishingTouch (U-Lead Systems, Taipei, Taiwan) and ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). The results of this quantification were used to normalize the results from subsequent labelling with FAT cDNA on the amount of RNA present on the blot. Thereafter, the blots were probed with the cDNA of FAT (see chapter 4) which was labelled using the Radprime DNA Labelling System from Gibco. Filters were prehybridized for one hour and hybridized overnight exactly as described in chapter 4. Non-specific binding was removed in several washing steps with increasing temperature and decreasing salt concentration. The final washing step consisted of 30 min incubation in 0.1 x SSC (SSC = saline sodium citrate, 0.15 M NaCl, 0.017 M $NA_3C_6H_5O_7$, pH 7.0)

containing 0.1% SDS at 56°C. Filters were exposed to X-ray film and to imaging screens for scanning and quantification (Phosphor Imager, ImageQuant; Molecular Dynamics).

RESULTS

Transfection of H9c2

Upon transfection of the H9c2 cells with PSG5-FAT and pMAMneo, 26 colonies were selected, isolated and cultured in selection medium. Fifteen of these colonies survived and could be maintained as cell-lines. These cell-lines were cultured and several batches were stored in liquid nitrogen. In addition cells were harvested for RNA, DNA and protein analysis. Differences in growth rate were observed between the cell-lines, which may be related to a variation in resistance to the selectable marker geneticin.

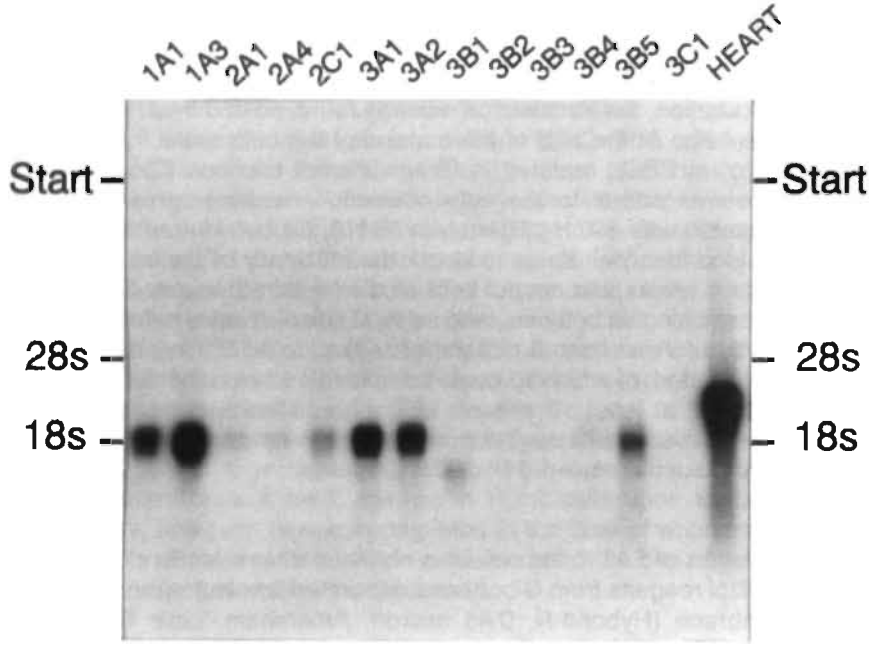

Figure 5.1 : *Northern blot analysis of RNA isolated from 13 cell-lines derived upon transfection of H9c2 cells with PSG5-FAT and pMAMneo probed with FAT cDNA. Rat heart RNA was included as control. The number-letter-number combinations indicate different cell-lines. 28S and 18S indicate the position of the signals for these ribosomal RNAs upon staining with methylene blue.*

Northern blot analysis

Northern blot analysis was performed with 5 μg of total RNA from 15 cell-lines obtained after transfection and from rat heart, which served as control RNA for FAT expression. RNA isolation, electrophoresis and transfer to Hybond-N was successful as judged by the staining of the RNA with methylene blue. Quantification of the ribosomal RNA bands as described in the methods section revealed that 28S signal was stronger than 18S signal

in all cases indicating that no major RNA degradation had occurred. The variation in amount of RNA present on the different lanes of the blot was about 25%.

The result of the northern blot analysis of RNA from 13 transfected cell-lines and rat heart RNA (control) using FAT cDNA as probe is shown in fig 5.1. A single strong signal for FAT is seen in the lane of rat heart in between the 28S and 18S signal, which is in accordance with a molecular mass of about 2.9 kb as was found in earlier studies (1,15). Several cell-lines also show a single strong signal, but this is found at a lower molecular mass of about the size of 18S (1.9 kb). It seems likely that differences between the mRNAs from the endogenous gene and the transfected cDNA with respect to the lengths of the 5' and 3' untranslated regions and the poly A tail are responsible for this finding. It is also clear from fig 5.1, that marked differences exist in the expression level of FAT, with some of the cell-lines expressing no measurerable amounts of FAT mRNA. Results of quantification of the FAT signal, normalized to the amount of RNA on the blot are presented in fig 5.2.

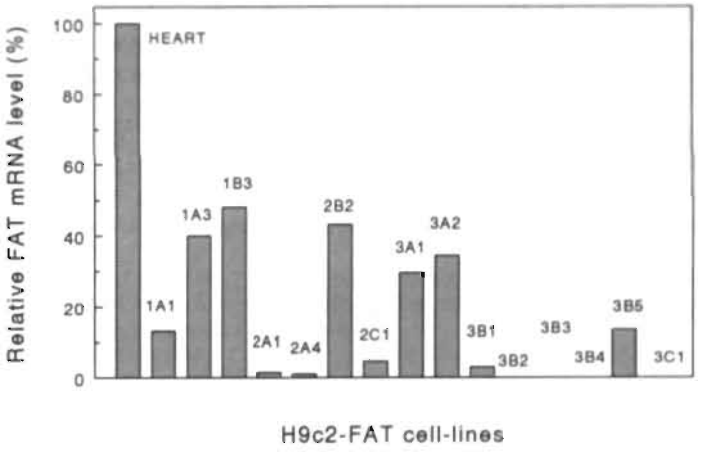

Figure 5.2 : *Quantification of the FAT mRNA levels of 15 different cell-lines obtained after transfection of H9c2 cells with FAT. The signal of FAT as found by northern blot analysis, was quantified using the phosphor imager results. The FAT mRNA level of each cell-line was normalized on the amount of RNA applied to the blot using the 18S signal (this was quantified by scanning the methylene blue staining). The FAT mRNA levels are presented relative to the FAT expression in rat heart, which is set at 100%.*

DISCUSSION

The aim of the present study was to investigate the role of FAT in the uptake of FA in muscle cells. H9c2 cells were chosen as a model because they have several characteristics of muscle cells, but have the advantage that they are immortalized. In addition, H9c2 cells do not express FAT in measurable amounts as was found using northern blot analysis (own unpublished observations). Therefore, stable transfection of FAT in these cells would provide a model to study the function of FAT in FA-uptake.

Finally, H9c2 cells were used in transfection studies before by other investigators (6,10), showing that the transfection technique was succesful in these cells.

After transfection, 15 cell-lines were obtained with different levels of FAT expression. Reasons for the differences in expression level of FAT could be:

1) The number of pSG5-FAT plasmids incorporated into the genome. Because co-transfection was used in this study, it is possible that only the pMAMneo plasmid is incorporated. However, this seems unlikely since the ratio of pSG5-FAT:pMAMneo was 20:1 (μg:μg).

2) The location of these plasmids in the genome. Although the FAT cDNA sequence on the pSG5-FAT plasmid is preceeded by the SV40 promotor, transcription can be influenced by the location of incorporation into the genome.

In addition to differences in FAT expression, some differences in growth rate were observed in the various cell-lines. These differences may, analogeous to the differences in FAT expression, be related to the number of pMAMneo plasmids incorporated in the genome and also to the site of incorporation. Growth rates were not quantified in these cell-lines, but some cell-lines expressing high levels of FAT also showed high growth rates. This finding is in correspondence with the idea that these cells have incorporated several copies of both pMAMneo and pSG5-FAT. On the other hand, FAT expression itself might have an influence on the growth rate of cells. Two cell-lines (1B3 and 2B2) showed high FAT mRNA levels, but grew relatively slow. In general, it is likely that growth rate will be related to expression of the neo gene, conferring resistence to geneticin. Future investigations will have to be performed to study these speculations.

FUTURE INVESTIGATIONS

Southern blotting

To investigate if the PSG5-FAT and pMAMneo plasmids are incorporated in the genome, and also to assess the number of incorporated plasmids, southern blot analysis should be performed with FAT cDNA and pMAMneo cDNA as probes. Results of this analysis will be compared with the mRNA levels found with the northern blot analysis. Another technique that could be used for this purpose is PCR. With primers that recognize part of the plasmids it is possible to check the cell-lines for incorporation of these plasmids. However, southern blot analysis seems easier to quantify and therefore would be the method of choice.

Western blotting

Since there is a lack of knowledge on the regulation of the cellular FAT protein level (transcriptional versus translational), it is possible that mRNA levels are a less reliable measure for the amount of protein present in the cells. Therefore it is important that also the protein level of FAT is studied in the different cell-lines obtained. At this moment, antibodies against FAT wich can be used successfully in western blot analysis are not available. However, since FAT is probably the rat homologue of human CD36 (see chapter 2), monoclonal antibodies raised against this protein may be useful in western blot analysis.

Cellular localization

FAT is a transmembrane protein, with probably one transmembrane region (see chapter 2). A function for this protein in the transport of FA across the plasmamembrane can only be imagined if the protein is indeed localized in this membrane. Therefore studies should be carried out to determine the cellular localization of FAT in the transfected cells. Immunocytochemical studies could be performed using the monoclonal antibodies against the CD36 as described in the former paragraph. These antibodies could then be used in confocal microscopy or in electron microscopy studies.

FA uptake studies

When the FAT transfected cell-lines are characterized using all the techniques mentioned above, the real aim of this study can be accomplished by measuring the FA-uptake in these cell-lines. Since we have different cell-lines with a wide range of FAT expression (at least on mRNA level), it will be possible to investigate whether there is a relation between the FAT expression level and the FA-uptake capacity.

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CHAPTER 6

CELLULAR PROTEIN RELEASE AS MARKER FOR MYOCARDIAL TISSUE INJURY:

A brief review

INTRODUCTION

Cells are surrounded by a biological membrane (plasma membrane) which separates the cytoplasm containing all cell organelles from the extracellular compartment. When the plasma membrane is damaged, cellular constituents will leak out of the cell and reach the interstitial and vascular space. Among the first released substances are cytoplasmic proteins which are not bound to intracellular structures such as the cytoskeleton. Detection of these proteins (in most cases through their enzymatic activity) in blood and/or urine from patients has been used since the beginning of this century to determine tissue injury (39).

Because the main issue of this thesis is the heart, we will focus primarily on myocardial cell damage upon ischemia. During ischemia, arterial blood flow is reduced and the affected tissue does not receive sufficient amounts of oxygen and energy substrates. At the same time waste products of cardiac metabolism are not completely carried away by the blood. When ischemia sustains cells will eventually die and cellular proteins are released into the interstitial space. Eventually, these proteins will reach the plasma via lymph flow or through the endothelial barrier.

The first report on protein release from ischemic myocardial tissue was published in 1954, and described the increase of serum activity of glutamic oxaloacetic transaminase after myocardial infarction (16). In subsequent decades a wide variety of proteins have been reported to be so-called biochemical markers for myocardial tissue injury (1).

MECHANISM OF PROTEIN RELEASE DURING ISCHEMIA

Since under physiological conditions the plasma membrane is virtually impermeable for macromolecules, massive protein release indicates membrane damage. It is generally accepted that protein release during ischemia is a consequence of a decrease in the energy level of the cell (15). The exact mechanism of protein release from ischemic cardiac tissue, however, is still incompletely understood (11,15,20). In the following part an attempt is made to describe the possible events leading to protein release from ischemic cardiomyocytes.

During ischemia, oxygen demand of the cardiomyocytes exceeds the supply of oxygen through the capillaries. This results in a reduction of oxidative phosphorylation, which in the case of total ischemia eventually will cease almost completely in about 10 seconds (11). The cardiomyocytes will stop contracting at the same time. Anaerobic glycolysis is the main source of ATP production in the flow-deprived cell. Since the supply of exogenous glucose is severely hampered, glycogen acts as glucose donor. The end product of anaerobic glycolysis is lactate and the production and accumulation of this substance lowers the cellular pH. Glycolysis is inhibited by an increase ratio of NADH to NAD^+, the low pH and, at a later stage, also by low ATP concentrations. In addition to the energy depletion, the cells accumulate waste products which are responsible for an increased osmolar load and subsequent cell swelling (11).

The reduced ATP concentration during prolonged ischemia affects several membrane ion channels, resulting in efflux of K^+ and influx of Na^+ (15). Part of the increase in osmolar load is caused by the inability of the cell to maintain the ion gradients across the sarcolemma (15,18). In a later stage the cell cannot exclude Ca^{2+} efficiently and the influx of Ca^{2+} is regarded as an important factor leading to cell death (10,15,26). The

influx of Ca^{2+} has been called "the final common pathway" of cell damage leading to cell death (26). However, others have stated that this is probably a too simplified view (15). It is worthwhile mentioning in this regard that the Ca^{2+} gradient across the cell membrane is largely maintained by ATP derived from glycolysis, and it was found that glycolysis is more important for the survival of cells than oxidative phosphorylation (13,14).

Upon an increase of intracellular Ca^{2+} concentrations several intracellular changes occur which could lead to membrane disruption and cell death. Duncan and Jackson (4,5) suggested that there are at least two different pathways leading to cell death upon an increase in intracellular Ca^{2+} concentration.

1) Activation of phospholipases A_2 and C, which result in the hydrolysis of membrane phospholipids and the formation of lysophospholipids and fatty acids. Both substances can influence membrane stability, but, in addition, arachidonic acid can form eicosanoids which have been suggested to play a role in cell damage (4,5). Contrastingly, eicosanoids are also reported to have beneficial effects on the ischemic and reperfused heart (35,37).

2) An increase of intracellular Ca^{2+} concentrations may affect the cytoskeleton by causing activation of several proteins including proteases which could break the connection between sarcolemma and cytoskeleton (22,28). One of the calcium activated proteases hypothesized to be involved in the cell damaging process is calpain (11). Recently, however, it was shown that this protein does not play an important role in the development of cell death in neonatal rat cardiomyocytes during metabolic inhibition (3). In addition to the activation of proteases, Ca^{2+} influences many other intracellular processes (i.e. contraction of the sarcomeres) which could increase the stress on the sarcolemma.

The combination of cell swelling and the decreased interaction of the cytoskeleton with the sarcolemma, caused by the increase in intracellular Ca^{2+}, result in the so-called blebbing of the sarcolemma, which is a vesicle formation characteristic for ischemia (15,18,21). An additional factor in the destabilisation of the sarcolemma is the loss of assymmetric distribution of the phospholipids over the inner and outer leaflet of the membrane (20). Together these processes could lead to the irreversibel sarcolemmal disruption and the loss of cell integrity.

REPERFUSION INDUCED INJURY

Reperfusion of ischemic heart tissue is without question essential for the affected tissue to survive (7,11). During reperfusion, oxygen and energy substrates again become available for the former ischemic tissue, and waste products of anaerobic metabolism are carried away to the systemic circulation or are further metabolized (11). However, it has been stated that reperfusion can have an additional damaging effect on the affected tissue, thus creating injury de novo (7). Others argued that the increased levels of protein release as observed during reperfusion are caused by an increase of the protein loss of cells that are already dead. Therefore, reperfusion would only accelerate protein loss and the cumulative release would be the same with or without reperfusion.

There are several indications that reperfusion can cause some additional injury to the myocardium (7). For example, adherence of polymorphonuclear leukocytes have been implicated in the reperfusion induced injury of the endothelium (17,34). Another adverse effect of reperfusion might be the induction of arrythmias, by stimulation of adrenergic

receptors, an increase in cyclic AMP or disturbances in lipid and/or ion homeostasis (7). It is still not clear, however, whether reperfusion itself can irreversibly damage cells that were only reversibly damaged during the ischemic period. Important for the clinical situation is the fact that early reperfusion after acute myocardial ischemia has always been shown to have benificial effects, and attempts to reperfuse the ischemic area should be performed as soon as possible (7).

REVERSIBEL AND IRREVERSIBLE CELL DAMAGE

One of the unclarified issues on cell damage and cell death is the (ir)reversibility of cell damage or "the point of no return" (15,25). As mentioned earlier, the influx of Ca^{2+} is recognized as a pivotal event in the development of cell damage leading to cell death (10,26). It is still not clear, however, whether the moment of influx of Ca^{2+} is really the initiation of the irreversibility of cell damage. The rupture of the sarcolemma, followed by release of macromolecules, is regarded by most investigators as a final sign of irreversible cell damage (11,15). After this, the cell looses important intracellular proteins and the ion gradients cannot be recovered. According to this hypothesis, it can be concluded that protein release from myocardial cells indicates cell death. However, Piper et al. reported enzyme release from isolated cardiomyocytes already during the reversible state of the cell damage process (25). This was concluded because these investigators observed a gradual increase in cytoplasmic protein release correlated with the depletion of energy reserves (25). A reasonable explanation for this phenomenon is that the vesicles formed by the blebbing process contain cytoplasmic fluid, and therefore also cytoplasmic proteins (15). In addition, some cytoplasmic constituents may be lost in the process of membrane resealing (tying-off) during the blebbing process (25). Calculations performed by Kristensen show that this reversibel protein release can only explain a minor part of the total protein release (15). In contrast with the findings of Piper et al. (25), other investigators found protein release to be correlated with cellular uptake of trypan blue (930 kDa) which indicates appreciable membrane permeability and cannot be explained by the blebbing phenomenon (19,36). These investigators concluded that complete release of soluble cytoplasmic proteins occurs as each individual cell dies. Thus cell death can be assessed by measuring release of soluble cytoplasmic proteins (19,36).

IS PROTEIN RELEASE A SELECTIVE PROCESS ?

Another unresolved question of protein release is whether small proteins are released earlier or faster during or following cell damage than proteins with a higher molecular mass. In 1967, Schmidt and Schmidt reported (27) that the rate of release of proteins from perfused liver was inversely proportional to their respective molecular masses. As was mentioned above, it was found by other investigators that protein release correlates with trypan blue uptake (19,36). Since the molecular mass of trypan blue (930 Da) is much smaller than that of the measured enzymes (80-140 kDa) it seems unlikely that differences would exist between release patterns of proteins with different molecular masses. This was also concluded by Murphy et al. (19) who measured the release of LDH (140 kDa), CK (80 kDa) and AST (100 kDa), and found a simultaneous release of the cytoplasmic part of these proteins. Mitochondrial CK (about 20 % of total CK activity)

and mitochondrial AST (about 70% of total AST activity) showed different release characteristics, and this was later confirmed by other investigators (2).

Recently the issue of possible preferential release of small proteins rather than larger proteins was addressed again, and it was shown that H-FABP (15 kDa) was released from damaged neonatal cardiomyocytes 2 hours earlier than CK (80 kDa) (32). This suggests that smaller proteins are released earlier from damaged cells than larger proteins. In the next chapter (**chapter 7**) this issue will be dealt with in more detail. We hypothesized that upon cellular damage all soluble cytoplasmic proteins would be released simultaneously from the cell. This was investigated by measuring the release of proteins with varying molecular mass (H-FABP, 15 kDa to LDH, 140 kDa), from neonatal cardiomyocytes during simulated ischemia and also during metabolic inhibition.

PROTEIN RELEASE AS A DIAGNOSTIC TOOL

Protein release from damaged cells can be used as a diagnostic tool to assess the occurrence and to estimate the extent of tissue injury. The latter can only be done if the major part of protein release is due to irreversibel cell damage (and thus cell death). Furthermore, in the clinical setting proteins are measured in blood plasma or serum and the proteins released from tissue cells like cardiomyocytes first have to reach the plasma compartment. Even if all proteins are released simultaneously from damaged cells there can be differences in rate of reaching the plasma compartment as this can occur through lymph drainage and/or directly through the endothelial barrier, as is shown in fig 6.1 (6).

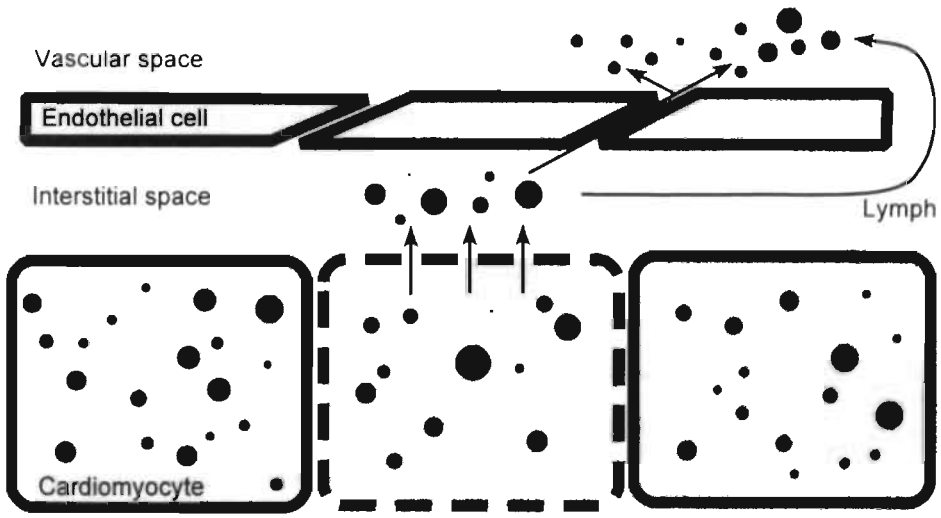

Figure 6.1 : *Schematic presentation of the possible transport routes of proteins released from damaged cardiomyocytes to the plasma compartment. Proteins can either directly cross the endothelial cell-barrier or they can be transported through lymph drainage.*

Important characteristic of a useful diagnostic marker are specificity for the tissue of interest, presence in the cytoplasm as a soluble protein, stability in blood plasma and relative ease of quantitation (1,9). One of the early markers for assessment of the occurrence and estimation of the extent of myocardial injury is H-FABP (6,12,33). This marker, however, is not specific for heart as skeletal muscles also contain H-FABP (23,24,38).

Myoglobin (18 kDa) is another early marker commonly used to determine myocardial tissue injury (8,29,30). Like H-FABP, this protein is also present in both heart and skeletal muscles. However, the myoglobin content of heart is lower than that in skeletal muscles (31), while the H-FABP content shows the opposite (24,38). We hypothesized that the ratio of myoglobin over H-FABP in the plasma compartment upon cell damage would reflect the ratio in the affected tissue. Therefore, this ratio could be useful in discriminating between heart and skeletal muscle injury. Results of a study to test this hypothesis using patients with myocardial injury (myocardial infarctions) and patients with skeletal muscle injury (surgery) are described in **chapter 8**.

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CHAPTER 7

RELEASE OF PROTEINS FROM ISOLATED NEONATAL RAT CARDIOMYOCYTES SUBJECTED TO SIMULATED ISCHEMIA OR METABOLIC INHIBITION IS INDEPENDENT OF MOLECULAR MASS

This chapter will be published in :
Van Nieuwenhoven FA, Musters RJP, Post JA, Verkleij GJ, Van der Vusse GJ and Glatz JFC (1996). Release of proteins from isolated neonatal rat cardiomyocytes subjected to simulated ischemia or metabolic inhibition is independent of molecular mass. J Mol Cell Cardiol. in press

ABSTRACT

This study addressed the question whether the molecular mass of proteins influences their release from isolated rat neonatal cardiomyocytes subjected to simulated ischemia (SI) or metabolic inhibition (MI). During these interventions cellular ATP content and the relative releases of several proteins, ranging in molecular mass from 15 to 140 kDa, were determined. After 180 min of normoxia, cellular ATP content was about 90% of the initial value, and cellular protein loss was about 1%. During either SI (180 min) or MI (120 min) the cellular ATP content decreased to less than 5% of the initial value. After 180 min of SI the release of soluble cytoplasmic proteins from the cells had increased to about 35%, and after 120 min of MI to about 90%. There were no major differences in the release pattern of four cytoplasmic proteins, during both SI and MI. A soluble mitochondrial and a partly mitochondrial protein, however, showed delayed release patterns. These data indicate that the release of proteins from damaged isolated neonatal rat cardiomyocytes is not related to the molecular mass of the proteins. It is concluded that protein release from damaged cardiomyocytes is not a sieving process in which small proteins are preferentially lost. In contrast, our data suggest that sarcolemmal disruption is a relatively fast process resulting in the simultaneous release of all soluble cytoplasmic proteins, irrespective of their molecular mass.

INTRODUCTION

The release of soluble cytoplasmic proteins from cells is widely used to monitor the occurrence and the extent of cell damage (6,9). The exact mechanism by which these proteins are released is still incompletely understood and there is controversy whether protein release is a selective process in such sense that small proteins are released earlier than proteins with higher molecular mass (5,10,12,15). Murphy et al. (10) reported the simultaneous and complete release of soluble cytoplasmic enzymes in the range of 80 to 140 kDa upon cell damage. However, Takahashi et al. described that heart-type fatty acid-binding protein (FABP, 15 kDa) was released 2 hours earlier from neonatal cardiomyocytes during hypoxia than was creatine kinase (CK, 80 kDa), and concluded that this was due to the differences in molecular mass (15).

We studied whether the initiation and rate of release of a protein from damaged cardiomyocytes is dependent on the molecular mass of that protein, by monitoring proteins with a much broader range of molecular masses (15 to 140 kDa) than in previous studies (10,15). Furthermore we investigated the effect of differences in cellular localization on the release of proteins.

In this study, isolated rat neonatal cardiomyocytes were used in which cell damage was induced by either a combination of anoxia, nutrient deprivation and volume restriction (simulated ischemia, SI) or incubation with inhibitors of energy metabolism (metabolic inhibition, MI) (11,13). The releases were monitored of the predominantly cytoplasmic proteins FABP (15 kDa), adenylate kinase (AK, 21 kDa), CK (80 kDa) and lactate dehydrogenase (LDH, 140 kDa) (3,5,10). The release of citrate synthase (CS, 100 kDa) as mitochondrial marker and aspartate aminotransferase (AST, 100 kDa) which is located in mitochondria (70%) as well as in the cytoplasm (30%), was also studied (7).

The present results indicate that not molecular mass of the proteins but intracellular localization determines the time-related pattern of their release upon simulated ischemia or metabolic inhibition.

MATERIALS AND METHODS

Cell culture
Rat neonatal cardiomyocytes were isolated and cultured as described previously (11). Before the experiment, cells were washed in buffer W containing 133 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl, and 5 mM glucose (pH 7.35). Incubations were performed at 37°C, in 35 mm or 60 mm Falcon culture dishes (Becton Dickinson, Plymouth, UK). As controls, cells were incubated aerobically in buffer W for 180 min.

Simulated ischemia (SI)
To simulate ischemia, air was replaced by water-saturated argon and the cells were nutrient deprived and volume restricted (1 ml of buffer W without glucose in a 35 mm dish) (11,16). Cells were subsequently incubated for 0, 30, 60, 90, 120, 150 or 180 min. After incubation the media were collected, diluted 1:1 in PPF (pasteurized plasma protein factor, Central Laboratory for Blood Transfusion Services, the Netherlands) to preserve enzyme activities, and frozen in dry ice. Cells were lysed by incubating them for 2 min in 1 ml of a 1:1 (vol/vol) mix of buffer W (without glucose) and NRS (nucleotide releasing agens for somatic cells, Lumac, Landgraaf, the Netherlands). From the cell lysate obtained, one aliquot was used to measure the ATP content directly, and another aliquot was treated identical as the media for measurement of protein release.

In a subset of experiments, 60 mm culture dishes containing 2 ml of medium were used, and cells were lysed in 2 ml buffer W containing NRS as described above. Cells and media in this subset were diluted 2:1 in a solution of 6% BSA in phosphate buffered saline (PBS), resulting in a final BSA concentration of 2%, to preserve enzyme activity.

Metabolic inhibition (MI)
Metabolic inhibition (MI) was induced by incubating the cells in a 35 mm Falcon dish for 0, 30, 60, 90 or 120 min in 1 ml buffer W without glucose but supplemented with 10 mM 2-deoxyglucose (DOG, Sigma, St Louis, MO, USA) and 1 mM iodo acetic acid (IAA, Sigma) (13). Subsequently, media and cells were treated identically as in the SI experiment.

Assay of ATP content
ATP was measured using a Lumac-2000 bioluminescence meter and a bioluminescent assay kit (Boehringer, Mannheim, Germany). The relative ATP level was determined by comparing the absolute values of the cell-sample with those of the control cells (0 min incubation).

Assay of protein release
FABP was measured using a non-competitive sandwich ELISA (17). LDH, CK, AK, AST and CS each were measured spectrophotometrically using a centrifugal analyzer (Hoffman-La Roche, Switzerland). LDH, CK and AST were measured using commercially

available test kits (Merck, Darmstadt, Germany), AK by using the method of Brolin (2), and CS as described by Shepherd and Garland (14). For individual incubation samples, the release of each of the proteins was determined by dividing its total amount in medium by the sum of the total amounts in medium and cells. Thus, the release is expressed as percentage of total cellular content or activity.

Statistics
Data are expressed as means±SD and a t-test for paired samples was used to monitor eventual statistically significant differences between the release of different proteins at one incubation time. A t-test for unpaired samples was used to determine statistically significant differences between the release of one specific protein after different incubation times. The level of significance was set at $p < 0.05$.

RESULTS

ATP content decreased to 90±2% of the initial level when cells were incubated under normoxic conditions for 180 min (fig 7.1). During the SI and MI experiments the ATP content of the cells decreased to a level below 5%, with the decrease being faster in the MI experiments than during SI (fig 7.1). During 180 min of normoxic incubation the release of proteins from neonatal cardiomyocytes was on the order of 1% for all proteins studied (data not shown).

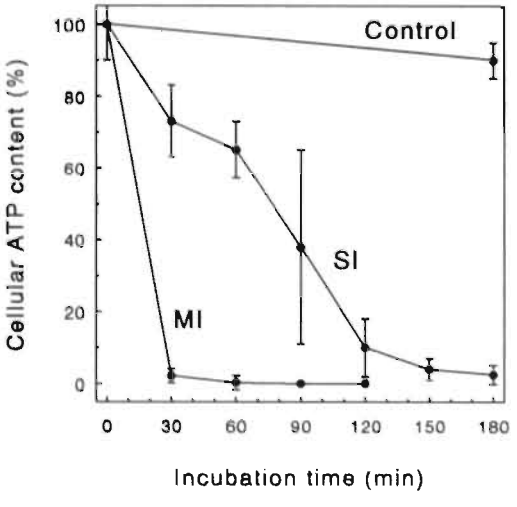

Figure 7.1 : *ATP content of neonatal cardiomyocytes subjected to 180 min of normoxia (control), 180 min of simulated ischemia (SI) or 120 min of metabolic inhibition (MI). Values are presented relative to the content at 0 min, and are expressed as mean ± SD of 3-6 incubations.*

Simulated ischemia (SI)
During SI hardly any protein was detected in the media in the first 90 min of incubation, but at 180 min about 35% of the cytoplasmic proteins (FABP, AK, CK and LDH) had been released (fig 7.2, top panel). The releases of all proteins were significantly elevated at 150 min of SI compared to 120 min of SI ($p < 0.05$). This indicates that the initiation of release had started before 150 min for all proteins studied. Of the mitochondrial

enzyme CS only 10 ± 5% was released after 180 min of SI, while AST, which is partly cytoplasmic and partly mitochondrial, was released for 20 ± 5% during this period. Both values are significantly different ($p < 0.05$) from those of each of the cytoplasmic proteins after the same time interval.

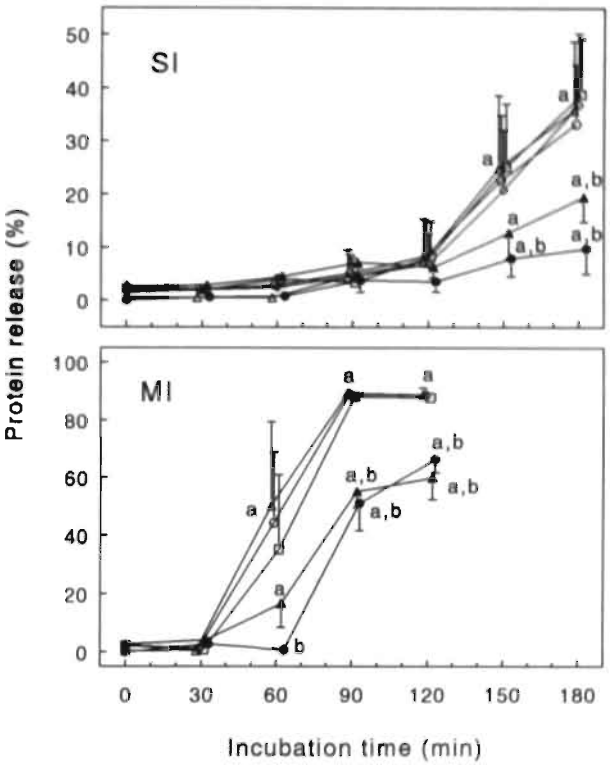

Figure 7.2 : *Release of proteins from neonatal cardiomyocytes during 180 min of simulated ischemia (SI, top panel), and during 120 min of metabolic inhibition (MI, lower panel). The release is presented relative to the total cellular protein content or activity, and is expressed as mean±SD for n = 5-9 incubations (except 0, SI30 and SI60, n = 3).* △ *FABP, fatty acid-binding protein;* o *AK, adenylate kinase;* ◇ *CK, creatine kinase;* □ *LDH, lactate dehydrogenase;* ▲ *AST, aspartate aminotransferase;* ● *CS, citrate synthase. a, significantly different from release at 120 min SI for all proteins, or from release at 30 min MI for all proteins except for CS (p < 0.05). b, significantly different from all cytoplasmic proteins (p < 0.05).*

Metabolic inhibition (MI)

During MI the release pattern of the soluble cytoplasmic proteins FABP, AK and LDH was similar and amounted to ca. 90% after both 90 min and 120 min (fig 7.2, lower panel). The release of all cytoplasmic proteins and of AST was significantly elevated at 60 min of MI as compared to 30 min MI ($p < 0.05$), while CS activity was not increased in the extracellular fluid at that time. This indicates that the initiation of release of all soluble cytoplasmic proteins started between 30 and 60 min of MI. Since the assay of CK was

directly influenced by the presence of IAA (data not shown) no reliable information on the release pattern of this enzyme could be obtained, and therefore, this enzyme is not included in fig 7.2, lower panel. The release of the mitochondrial enzyme CS and also that of the partly mitochondrial enzyme AST showed a delayed release pattern amounting in about 60% release after 90 and 120 min of MI, which is significantly different from that found for the cytoplasmic proteins ($p < 0.05$).

Detailed comparison of FABP and LDH release

For both SI and MI a considerable variation was found in the amount of protein release when individual dishes of the same incubation time were compared. However, within these individual dishes cytoplasmic proteins showed similar release values, indicating that this variation is not related to molecular mass. To study this in more detail, we compared the release of FABP (smallest cytoplasmic protein studied, 15 kDa) and LDH (largest protein, 140 kDa) from the individual incubations of SI and MI in a scatter diagram (fig 7.3, top panel). We included only those release values from SI and MI between 5 and 50% to exclude incubations where either minute amounts of protein release had occurred or those where protein release was almost complete. In addition, we compared FABP release to CS release (fig 7.3, lower panel). The curve found by linear regression for LDH release as function of FABP release was not significantly different from the line of identity (dotted line). Contrastingly, using the same method, a significant difference was found between FABP release and CS release (fig 7.3, lower panel).

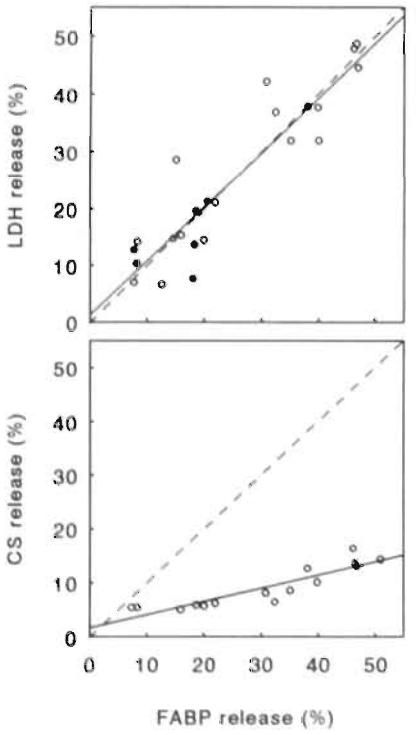

Figure 7.3 : *Scatter diagram for the release of FABP with that of LDH (top panel) and CS (bottom panel) in individual incubations of neonatal cardiomyocytes subjected to either simulated ischemia (o) or metabolic inhibition (●). Incubations were included in this diagram irrespective of time interval, when the release of both proteins was > 5% and < 50% of total cellular content. Consequently only 5 values of MI were included in the scatter diagram of FABP/LDH, while for FABP/CS only SI values could be used. The dotted line is line of identity (y=x) and the solid lines are the relations calculated by linear regression: LDH as function of FABP, y=0.95x+2.7; CS as a function of FABP, y=0.25x + 1.6.*

DISCUSSION

In this study the protein release from neonatal cardiomyocytes was measured during simulated ischemia (SI) and metabolic inhibition (MI). Cellular ATP content decreased much faster during MI than during SI. Since the release of proteins started earlier during MI (between 30 and 60 min) than during SI (after 90 min), these data confirm that loss of cell integrity is related to the cellular ATP content (8,9,12).

There were no major differences in release pattern between the individual cytoplasmic proteins, varying in molecular mass between 15 to 140 kDa, during either SI and MI. This indicates that no major differences exist in the initiation and rate of release of soluble cytoplasmic proteins from neonatal cardiomyocytes under these damaging conditions. The mitochondrial enzyme CS and the enzyme AST (30% cytoplasmic, 70% mitochondrial), however, showed release values significantly lower than any of the cytoplasmic proteins at 180 min of SI and after 90 and 120 min of MI. Together, these data confirm the earlier observations that the release pattern of a protein depends largely upon its intracellular localization (1).

The present results indicate that during the protein release phase, the sarcolemma does not act as a sieve through which small proteins are preferentially lost. Our data extend the finding of Murphy et al. (10) to lower molecular mass proteins, but contradict the data published by Takahashi et al. (15). In the latter study no detectable CK (80 kDa) release was found during the first 3 hours of hypoxic incubation of neonatal cardiomyocytes, whereas FABP (15 kDa) was found in the media already after 1 hour of hypoxia. The reason for this discrepancy is largely unknown, but may be caused by the instability of the enzymatic activity of CK, or the use by these investigators of a less specific assay method for FABP.

The consequence of our findings for the use of proteins as plasma marker for tissue injury is, that upon cell damage all soluble cytoplasmic proteins are most likely released simultaneously into the interstitial space. Thereafter, the speed of reaching the plasma is governed by the permeability of the endothelial barrier and by lymph drainage. In this process smaller proteins like FABP could reach the plasma compartment earlier than larger proteins like LDH, as was suggested earlier (4).

In conclusion, the results of this study support the concept that the initiation and rate of release of soluble cytoplasmic proteins from damaged cells is independent of their molecular mass. However, proteins from other cellular compartments (i.e. mitochondria) show different release characteristics.

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CHAPTER 8

DISCRIMINATION BETWEEN MYOCARDIAL AND SKELETAL MUSCLE INJURY BY ASSESSMENT OF THE PLASMA RATIO OF MYOGLOBIN OVER FATTY ACID-BINDING PROTEIN

This chapter was published as
Van Nieuwenhoven FA, Kleine AH, Wodzig KWH, Hermens WT, Kragten HA, Maessen JG, Punt CD, Van Dieijen MP, Van Der Vusse GJ and Glatz JFC (1995). Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein. Circulation 92, 2848-2854

ABSTRACT

Myocardial and skeletal muscle contain substantial amounts of myoglobin (18 kDa) and fatty acid-binding protein (FABP) (15 kDa). Upon muscle cell damage both proteins are released rapidly into plasma, and are used as early biochemical markers of muscle injury. We studied whether the ratio of myoglobin over FABP in plasma can be used to distinguish myocardial from skeletal muscle injury.

Myoglobin and FABP were assayed immunochemically in tissue samples of human heart and skeletal muscles, in serial plasma samples from 22 patients with acute myocardial infarction (AMI), and in serum samples from 9 patients undergoing aortic surgery and plasma samples from 10 patients undergoing cardiac surgery. The latter interventions were suspected to cause marked injury of skeletal muscles alone (aortic surgery), or in combination with myocardial injury (cardiac surgery). In human heart tissue the ratio of the contents of myoglobin over FABP was 4.5 and in skeletal muscle varied from 21 to 73. After AMI the plasma concentrations of both proteins were elevated significantly between ≈ 1 and 15-20 hours after first onset of symptoms. In this period the ratio of myoglobin over FABP in plasma was constant both in a subgroup of 9 patients receiving thrombolytics and a subgroup of 10 patients not receiving thrombolytics, and amounted to 5.3 ± 1.2 (mean ± SD; 219 samples), which is similar to the ratio monitored in heart tissue. In 7 out of 9 patients who underwent aortic surgery the serum concentrations of both proteins were significantly elevated between 6 and 24 hours after surgery, and the ratio of myoglobin over FABP was 45 ± 22 (mean ± SD; 26 samples), which is in accordance with the ratio in human skeletal muscles and significantly different from that found in plasma from patients with AMI ($p < 0.001$). In 10 patients with cardiac surgery the ratio increased postoperatively from 11 ± 5 to 32 ± 14 (means ± SD, n=10) at 24 hours after surgery, indicating more rapid release of protein from injured myocardium than from skeletal muscles.

The ratio of immunochemically assayed concentrations of myoglobin over FABP in plasma from patients with muscle injury reflects the ratio found in the tissue of origin. Since this ratio is significantly different between heart (4.5) and skeletal muscles (20-70), its assessment in plasma allows the discrimination between myocardial and skeletal muscle injury in humans.

INTRODUCTION

Biochemical markers of myocardial injury are helpful tools in differentiating patients with and without acute myocardial infarction (AMI), thereby defining the small percentage (10% to 20%) of patients with symptoms consistent with ischemia who indeed had an AMI (1). Important characteristics determining the utility of a biochemical marker are its cellular localization, aqueous solubility, clearance from the circulation, specificity for myocardial tissue and detectability in plasma (1).

Recently heart-type fatty acid-binding protein (FABP) has been introduced as a plasma marker for the early assessment of myocardial tissue injury (7,13,14,22) and estimation of infarct size (7) in humans. This small (15 kDa) cytoplasmic protein is abundant in cardiomyocytes and is assumed to be involved in myocardial lipid homeostasis (8). Heart-type FABP is distinct from other types of FABP such as those found in liver and intestine (8,26). The plasma concentration of (heart-type) FABP is significantly increased within

3 hours after AMI (13), similar to that of myoglobin (18 kDa) which previously has been described as early biochemical marker for myocardial injury (9,19,20).

Both (heart-type) FABP and myoglobin are low-molecular mass, cytoplasmic proteins present not only in the heart but also in skeletal muscle (8,21). This feature makes it difficult to discriminate between heart and skeletal muscle injury when plasma levels of these proteins are used as markers for loss of muscle cell viability. However, the myoglobin content of human heart is lower than that of skeletal muscle (21), while studies in humans (15) and rats (27) have shown that the FABP content is at least twice as high in heart as in skeletal muscles. Therefore we hypothesized that when the ratio of the contents of myoglobin and FABP in heart and skeletal muscle would differ significantly, and upon muscle injury both proteins are released into and cleared from the blood to a similar extent, the ratio of the increased plasma concentrations of myoglobin and FABP would be a useful index to identify the type of injured muscle.

The aims of this study were i) to investigate whether the ratio of myoglobin over FABP in human myocardial tissue is substantially different from the ratio in skeletal muscle tissue and, if this is the case, ii) whether the assessment of this ratio in plasma can be used to discriminate between myocardial and skeletal muscle injury. To this end the myoglobin and FABP contents were assessed in samples from human heart and various types of human skeletal muscles. Subsequently, the myoglobin and FABP concentrations were assessed in blood samples from patients after AMI, and from patients after either aortic or cardiac surgery. These latter patients were suspected to have skeletal muscle damage alone (aortic surgery) or in combination with myocardial muscle damage (cardiac surgery). During aortic surgery the aorta is clamped just beneath the renal arteries, rendering the lower part of the body ischemic, and thus leading to a certain degree of skeletal muscle injury. Previous studies have shown that surgery alone can already lead to detectable skeletal muscle injury (29). The ratios of the blood concentrations of the two biochemical markers then were compared for myocardial injury, skeletal muscle injury, and the occurrence of a combination of myocardial and skeletal muscle injury.

MATERIALS AND METHODS

Patients with acute myocardial infarction (AMI)

Twenty-two patients with an initial clinical diagnosis of AMI were included in this study, divided into three subgroups.

1) A subgroup of 10 patients not receiving thrombolytic therapy (8 female, 2 male; age 71 ± 10 years, mean ± SD; ischemia located anteriorly in 8 cases and inferioposteriorly in 2 cases).

2) A subgroup of 9 patients receiving Streptokinase® (1.5 million units) (1 female, 8 male; age 58 ± 10 years, mean ± SD; ischemia located anteriorly in 2 cases and inferioposteriorly in 7 cases).

3) Three special cases (all males with inferior myocardial infarction) being a patient (45 years) who underwent cardioversion, a patient (59 years) who developed a recurrent AMI, and a patient (68 years) with AMI in combination with severe renal insufficiency.

AMI was positively diagnosed when patients showed elevation of more than one millimetre of the ST-segment in the recorded cardiogram and typical presentation of chest pain (often combined with radiation of pain to the left arm), in combination with transpiration, nausea, and/or shortness of breath. Reasons for omitting treatment with

thrombolytic agents were increased risk of bleeding or previous coronary bypass surgery. Starting from admission of the patients to the coronary care unit of the hospital (2.1 ± 1.1 hours and 2.6 ± 1.0 hours after onset of symptoms for patients not receiving and receiving thrombolytic therapy, respectively; means ± SD), blood samples were taken every hour during the first ten hours (except for the patient with renal insufficiency every three hours). Thereafter blood samples were taken every 6 or 12 hours, in accordance with the hospital routine. The blood samples were collected in glass tubes coated with ethylenediamine tetraacetic acid (EDTA) (Sherwood Medical, St. Louis, MO, USA) and centrifuged at 1500 x g for 10 min. Plasma was collected and stored at -70°C until use.

Patients undergoing aortic or cardiac surgery

We studied a group of 9 patients undergoing aortic surgery (1 female, 8 male; age 72 ± 11 years, mean ± SD) and a group of 10 patients undergoing cardiac surgery (1 female, 9 male; age 63 ± 8 years, mean ± SD). The aortic surgery patients had either an aneurysm of the abdominal aorta (5 patients) or occlusive arterial disease (4 patients), and were given protheses of part of the abdominal aorta, the aorta bifurcation or the common iliac artery. For this, the aorta was clamped distal of the renal arteries, rendering the lower part of the body ischemic (period of ischemia 73 ± 55 min; mean ± SD). This intervention was expected to lead to significant skeletal muscle damage. None of the patients had a recent history of myocardial injury.

The other group consisted of patients with left ventricular dysfunction undergoing coronary bypass surgery and/or valve replacement surgery. Patients were recruted from those operated upon in the period July 1994 to February 1995, and were selected on the basis of a post-surgery increase in plasma activity of creatine kinase isoenzyme MB of more than 20 U/l, indicating the occurrence of significant myocardial injury. The period of ischemia was 74 ± 37 min (mean ± SD)

Serial blood samples obtained immediately before and after the surgery were collected in CORVAC separator tubes (aortic surgery) or glass tubes coated with EDTA (cardiac surgery) (Sherwood Medical, St. Louis, MO, USA). After centrifugation at 1500 x g for ten minutes, serum or plasma, respectively, was collected and stored at -70°C until use.

Study approval

For each substudy applies that the experimental protocol was thoroughly explained to the patients, and informed consent was obtained. The study protocols were approved by the Medical-Ethical Committees of the Academic Hospital Maastricht and the De Wever Hospital Heerlen.

Tissue samples

Tissue samples of intact human heart and various skeletal muscles were obtained after autopsy (performed within 12 hours after death) from the Academic Hospital Maastricht. The samples were stored at -20°C until use. All steps of the tissue homogenization procedure were performed at 4°C or on ice. The tissue samples were homogenized (5%, w/v) in phosphate buffered saline (PBS, pH 7.4) containing 3% (w/v) bovine serum albumin (Sigma, St. Louis, MO, USA), using an Ultra-Turrax homogenizer (IKA Werke, Breisgau, Germany). Thereafter the samples were centrifuged at 2000 x g for 15 minutes and the supernatants stored at -70°C until use.

Sandwich ELISA for FABP

Bovine serum albumin (BSA, A7888), horseradish peroxidase (HRP, P8375), N-hydroxysuccinimidobiotin (NHS-d-biotin, H1759) and o-phenylenediamine dihydrochloride (OPD, P1526) were obtained from Sigma (St. Louis, MO, USA).

FABP was determined in plasma, serum and supernatants of tissue sample homogenates using an enzyme linked immunosorbent assay of the antigen capture type (sandwich ELISA). This assay was developed essentially according to that described by Börchers et al. (3) for bovine heart FABP and that described by Vork et al. (27) for rat heart FABP.

In short, rabbit antibodies directed to human heart-type FABP were coated on 96 wells microtiterplates (Falcon type 3912, Becton Dickinson, Oxnard CA) in 0.1 M carbonate buffer pH 9.6 at 37°C for 2 hours. All further steps were performed at room temperature in PBT [phosphate-buffered saline, pH 7.2, supplemented with 0.1% (w/v) BSA and 0.05% (v/v) Tween-20]. In between every step the plate was washed 5 times with PBT. After coating and washing, 50 µl of sample or standard was incubated for 90 min allowing the FABP to bind to the antibodies attached to the plates. Then a second antibody, either directly conjugated with HRP or biotinylated, was incubated for 90 min. In case conjugated antibody was used, 100 µl substrate mixture, containing 20 mM o-phenylenediamine (OPD) and 6 mM H_2O_2 in 0.1 M citrate buffer (pH 5) was added to each well. The biotinylated antibody required an additional incubation for 60 min with streptavidine-HRP (Pierce, Rockford, IL, USA). In both cases the enzyme reaction was stopped after 5-10 min with 50 µl 2 M H_2SO_4 and the absorbance at 492 nm measured using a microplate reader (Titertek Multiskan MKII). Detection limit of the assay was 0.5 µg/l (25 pg/well). Recovery experiments (n=11) using normal human plasma spiked with purified human heart FABP yielded an average recovery of 93%. The interassay coefficient of variation was on the order of 7%.

Determination of myoglobin

Myoglobin was determined in plasma and tissue samples using a turbidimetric immunoassay (Turbiquant immunoassay, code No OWNL, Behring, Hoechst Holland, Amsterdam) on a Turbitimer analyzer (Behring, Hoechst Holland). Assay of myoglobin was performed according to the method of Delanghe et al. (6). Turbiquant Myoglobin is a freeze-dried reagent consisting of polystyrene latex particles (size 100 nm) coated with rabbit anti-human myoglobin. The lyophilized reagent is resuspended with 10 ml of citrate buffer (pH 7.8). In the assay, the cuvette is filled with 50 µl plasma, serum or tissue homogenate, and 500 µl of suspended latex particles. Dilutions of plasma, serum and tissue homogenate were made in saline (0.9% NaCl). The myoglobin concentration is determined by turbidimetric measurement of the maximum reaction velocity (peak-rate method). The bar code on the package insert contains the calibration information needed for the assay. These data are stored by the instrument and can be used as long as the reagent lot number remains unchanged. Detection limit of the method is 50 µg/l. The pre-programmed measuring range covers myoglobin concentrations from 50-650 µg/l.

Internal quality control was performed using the human Apolipoprotein Control Serum CHD (Behring, OUPH 06/07, lot no. 063617, assigned value 95 µg/l and confidence limit 81-109 µg/l). Day to day variation was obtained by measuring the control serum on 22 subsequent days resulting in a mean concentration of 97.4 µg/l and a day to day variation of 4.8%.

Calculation of myoglobin/FABP ratio
The ratio of myoglobin over FABP in cardiac and skeletal muscle tissue was calculated directly from the tissue contents of these proteins. The ratio of myoglobin over FABP in plasma or serum upon muscle injury was calculated from the increased levels of myoglobin and FABP. The basic levels of both proteins were subtracted from the plasma or serum levels measured. For FABP, individually measured basic values were used. For the AMI patients the basic value was the FABP concentration measured in the first sample taken after arrival in the hospital or, in case this sample already showed a significantly raised FABP level (>19 μg/l, ref (13)), the plasma level more than 36 hours after AMI, or the average FABP basic level of 9 μg/l was used (13). The basic FABP levels of patients undergoing aortic or cardiac surgery were determined by measuring blood samples before surgery. Because the practical lower detection limit of the currently used myoglobin assay is 50 μg/l, the basic plasma level of myoglobin was assumed to be 30 μg/l (4,5,9,19).

The ratios of myoglobin over FABP were calculated only for those samples in which both proteins were raised at least twice above their basic value. For this reason calculations were not performed for some time points.

Statistical analysis
Data are expressed as mean±SD as indicated. Release curves of proteins into plasma or serum and curves of ratios of myoglobin over FABP are presented as mean±SEM for sake of clarity. Pearsons correlation coefficient was calculated to show relations between the myoglobin and FABP contents of human heart and skeletal muscles. A t-test for independent samples was used to monitor eventual statistically significant differences. The level of significance was set at $P < 0.05$.

RESULTS

Tissue contents of FABP and myoglobin
The FABP and myoglobin contents were measured in five different areas of the human heart and in various types of skeletal muscle (table 8.1). The FABP content of left ventricular tissue is similar to that found in an earlier study (0.56 ±0.07 mg/g wet weight; mean ± SD for 17 individuals) (7). For the calculation of the average contents of both proteins in the total heart, the differences in total mass of the left and right ventricle were taken into account. The weight ratio of right/left ventricle was taken as 1/3 (11). It is apparent from table 8.1 that the FABP values (normalized on gram wet weight of muscle) are higher in heart than in any of the skeletal muscles examined, while in most cases the myoglobin levels show the opposite. This type of FABP (heart or muscle type) is also expressed in some other organs and tissues such as smooth muscle, kidney and mammary gland, but always in markedly lower quantities than found in heart and skeletal muscles (28,31).

Table 8.1 : *Myoglobin and FABP contents of human heart and skeletal muscles. Data represent means ± SD for the indicated number of individuals, and are expressed as mg myoglobin or FABP per gram wet weight of tissue. *Calculated from the contents of the left and right ventricle (see text).*

Tissue	n	Myoglobin content (mg/g ww)	FABP content (mg/g ww)	Ratio Myoglobin/FABP (mg/mg)
HEART				
Left Ventricle				
Anterior wall	5	2.8 ± 0.7	0.61 ± 0.06	4.6 ± 1.0
Side wall	5	2.5 ± 0.8	0.54 ± 0.14	4.6 ± 0.8
Posterior wall	5	2.2 ± 0.5	0.53 ± 0.09	4.1 ± 0.9
Septum	3	2.2 ± 1.0	0.51 ± 0.21	4.4 ± 0.4
Right Ventricle	5	1.9 ± 0.5	0.41 ± 0.06	4.7 ± 1.0
Total heart*		2.4 ± 0.5	0.52 ± 0.06	4.5 ± 0.8
SKELETAL MUSCLE				
Brachialis	1	6.7	0.14	48
Gastrocnemius	1	2.4	0.040	60
Psoas	2	3.7, 3.7	0.088, 0.168	42, 22
Rectus femoris	4	2.9 ± 0.6	0.068 ± 0.022	45 ± 9
Soleus	2	3.0, 1.4	0.041, 0.064	73, 21
Vastus lateralis	3	3.5 ± 0.3	0.071 ± 0.016	51 ± 8

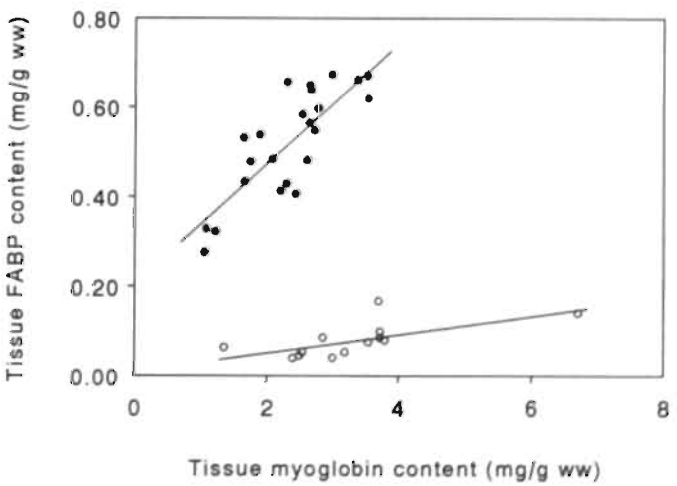

Figure 8.1 : *Relation between myoglobin and FABP content in individual samples from different areas of human heart (●, n=23) and various types of skeletal muscle (o, n=13). A significant correlation is found for the contents of myoglobin and FABP among the muscle samples from heart ($r = 0.82$; $p < 0.001$) and those from skeletal muscle ($r = 0.66$; $p = 0.014$).*

The ratio of myoglobin over FABP appeared to be rather constant among the various parts of the human heart and was found to be about 10 times lower in heart than in skeletal muscles (table 8.1). In addition, a significant correlation was found for the contents of myoglobin and FABP in the individual muscle samples, both from heart ($r=0.82$; $n=23$; $p<0.001$) and from skeletal muscles ($r=0.66$; $n=13$; $p=0.014$). This relationship between the myoglobin and FABP contents in the individual heart and skeletal muscle samples is shown in fig 8.1.

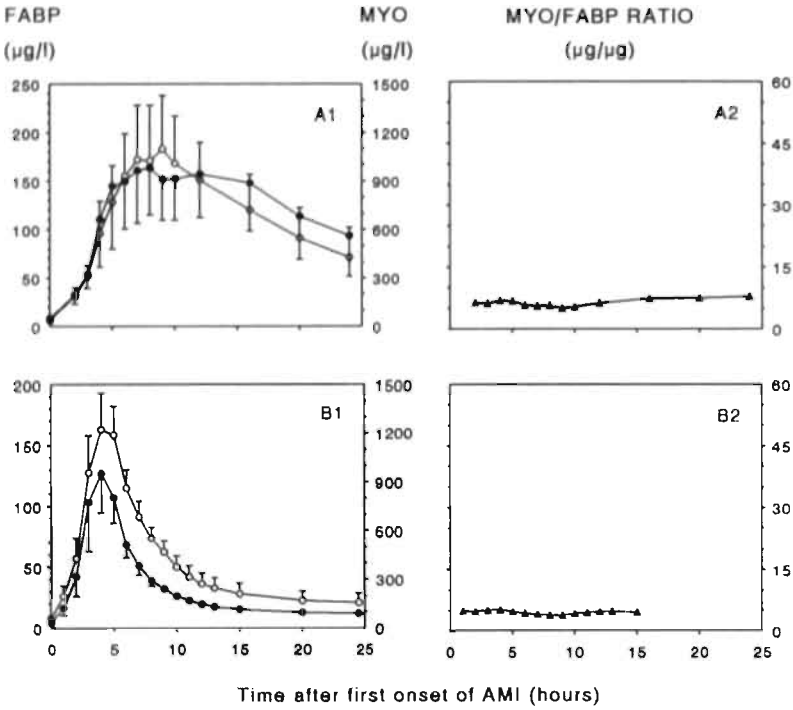

Figure 8.2 : *Mean plasma concentrations of myoglobin (●) and FABP (o) (left panels) and the myoglobin/FABP ratio (▲) (right panels) in 10 patients after acute myocardial infarction (AMI) and not receiving thrombolytic therapy (A), and in 9 patients after AMI and receiving thrombolytic therapy (B). Data refer to means ± SEM. Myoglobin/FABP ratios on each time point were calculated as means of the ratios found for each patient; the small standard error, however, is not larger than the size of the symbols used.*

Protein release after AMI

In 19 patients with AMI, 9 receiving and 10 not receiving thrombolytic therapy, the plasma concentrations of myoglobin and FABP were measured in serial samples obtained during the first 25 hours after first onset of symptoms. All patients showed a marked and simultaneous release of myoglobin and FABP into plasma within a few hours after onset of pain, with peak values being reached at about 8 hours (patients not receiving thrombolytics) (fig 8.2A1) and about 4 hours after first onset of symptoms (patients receiving thrombolytics) (fig 8.2B1). In both groups of patients the ratio of myoglobin over FABP appeared constant during the time of elevated plasma concentrations, amounting

to 6.2 ± 1.0 (mean ± SD, 126 samples; range 2.2-10.5) for 10 patients not receiving thrombolytics, and 4.4 ± 1.4 (mean±SD, 93 samples; range 2.1-6.6) for 9 patients receiving thrombolytics (fig 8.2A2 and 8.2B2).

Protein release after aortic or cardiac surgery

Myoglobin and FABP concentrations were measured in blood samples obtained immediately before surgery and during the first 24 hours after surgery. For these patients the mean release curves are shown in fig 8.3. The ratio of myoglobin over FABP was calculated for those samples in which both myoglobin and FABP concentrations were raised at least twice their basic values. In case of aortic surgery 7 of the 9 patients showed release of both proteins to a level twice above their individual basic level in samples between 6 and 24 hours after surgery. The mean serum ratio of myoglobin over FABP varied from 35 to 50 (fig 8.3A2), and for all samples examined amounted to 45 ± 22 (mean±SD, 26 samples, 7 patients). In case of cardiac surgery all 10 patients showed a marked increase in plasma concentrations of both proteins (fig. 8.3B1). In these patients the ratio of myoglobin over FABP was 11.3 ± 4.7 at 0.5 hours after surgery, decreased to 6.7 ± 3.7 at 8 hours after surgery and then increased again to 32.1 ± 13.6 at 24 hours after surgery (values at 8 and 24 hours each significantly different from value at 0.5 hours; $P < 0.05$) (fig 8.3B2).

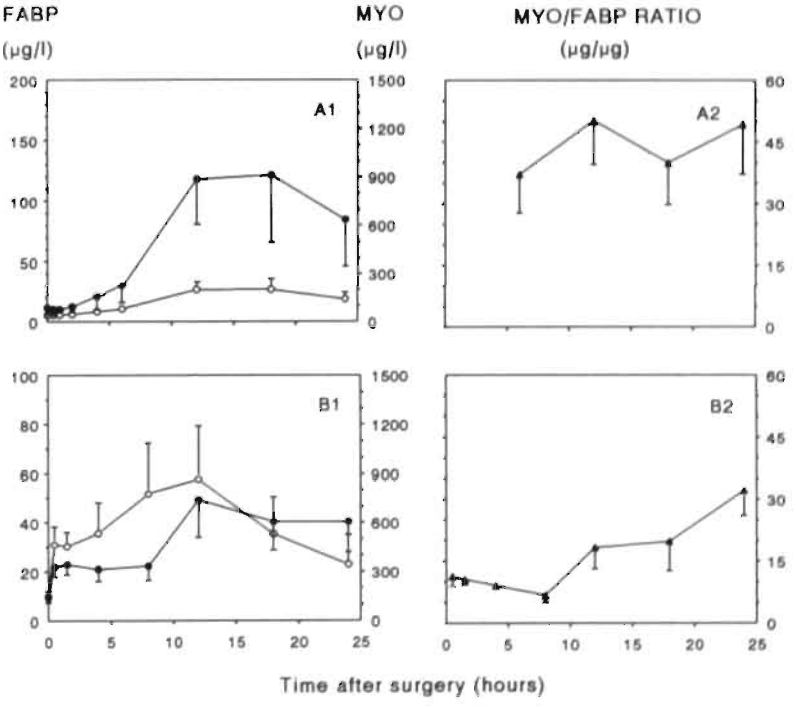

Figure 8.3 : *Mean serum or plasma concentrations of myoglobin (●) and FABP (o) (left panels) and the myoglobin/FABP ratio (▲) (right panels) in 9 patients after aortic surgery (A) and in 10 patients after cardiac surgery (B). Data on myoglobin/FABP ratios on each time point were calculated as means of the ratios found for each patient. Data refer to means ± SEM.*

Special cases
In order to assign the clinical significance of the plasma ratio of myoglobin over FABP for patients with myocardial infarction, we also studied three special cases. One patient underwent cardioversion (defibrillation) around 4 hours after the first onset of symptoms of AMI, a treatment that could very well have resulted in skeletal muscle damage (most likely of the intercostal and pectoral muscles). The release curves of myoglobin and FABP of this defibrillated patient (fig 8.4A1) are different from the mean release curves of the non-defibrillated patients (fig 8.2B1). Interestingly, the plasma ratio of myoglobin over FABP increased from 8 at 4 hours after AMI to over 50 at 24 hours after AMI (fig 8.4A2).

Another patient developed a recurrent myocardial infarction soon (< 10 hours) after the initial AMI. The appearance of this recurrent infarction is reflected clearly in the plasma curves for myoglobin and FABP (fig 8.4B1). However, the plasma ratio of myoglobin over FABP is constant in time, amounting to 4.6 ± 0.8 (mean ± SD, 8 samples) (fig 8.4B2). A third patient suffered from AMI in combination with severe renal insufficiency, which caused the plasma concentrations of both myoglobin and FABP to remain elevated during the entire period of blood sampling (fig 8.4C1) but, however, did not affect the myoglobin over FABP ratio in this time interval; the ratio was 2.9 ± 0.4 (mean ± SD, 12 samples) (fig 8.4C2).

DISCUSSION

One of the primary characteristics which a biochemical marker of myocardial damage should possess is a specificity for cardiac tissue (1). Myoglobin and FABP each are early biochemical markers present in substantial amounts not only in the aqueous cytoplasm of cardiomyocytes, but also in that of skeletal muscles (15,21,27), thus limiting their use as plasma markers of myocardial injury. However, the present study shows that the ratio of the tissue contents of myoglobin and FABP is markedly different between heart and skeletal muscles, and that in patients with muscle injury the ratio of the plasma concentrations of myoglobin and FABP reflects that of the injured tissue, indicating the use of this ratio to discriminate myocardial from skeletal muscle tissue injury.

The immunochemically assessed FABP content is found to be higher in human heart than in various skeletal muscles while the myoglobin content in the majority of cases shows the opposite. These differences are best reflected in the ratios of the tissue contents of myoglobin over FABP, being 4.5 for heart and 21-73 for the skeletal muscles studied, which covers the entire range of types of skeletal muscles.

Myocardial infarction
The myoglobin over FABP ratio found in plasma after myocardial injury (AMI) was constant during the entire sampling period and for patients not receiving thrombolytics amounted to 6.2 ± 1.0 (mean ± SD, n=126, 10 patients) and for those receiving thrombolytics to 4.4 ± 1.4 (mean ± SD, n=93, 9 patients). These mean ratios are not significantly different, despite the differences in shape of the release curves found for the two groups of patients. It is important to note that the ratio of myoglobin over FABP found in plasma agrees with the ratio found in heart tissue. In serial blood samples obtained from a larger, but less frequently sampled group of patients with AMI and who had been treated with thrombolytic agents (7), we also found the plasma ratio of myoglobin over

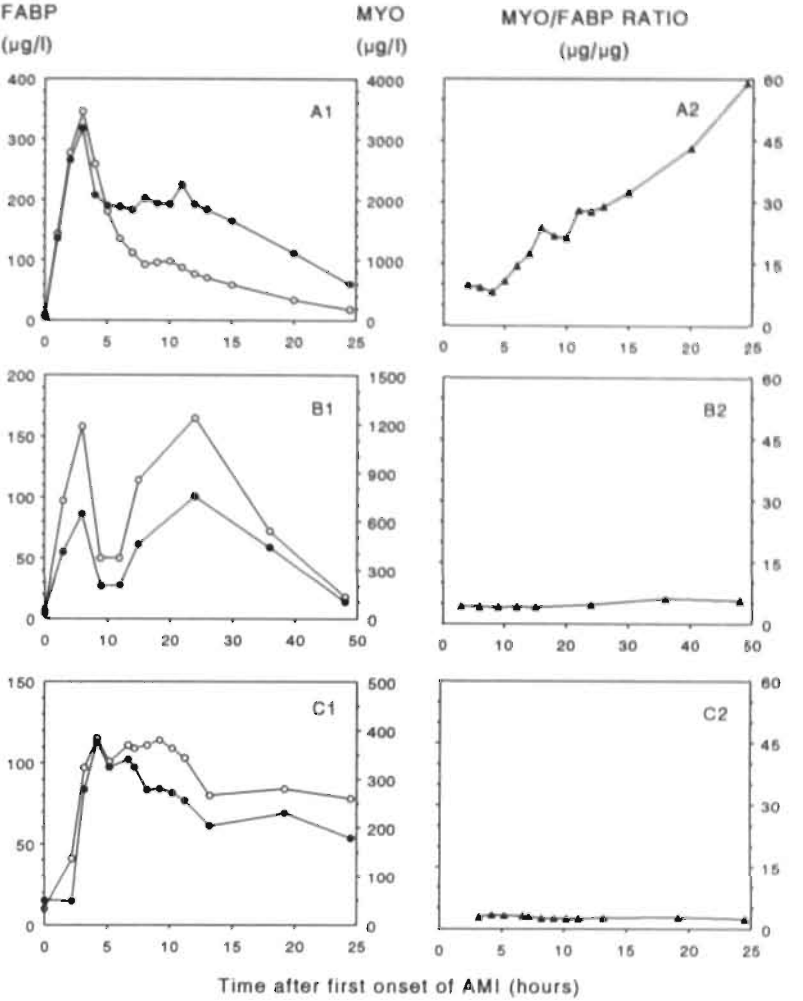

Figure 8.4 : *Mean plasma concentration of myoglobin (●) and FABP (o) (left panels) and the myoglobin/FABP ratio (▲) (right panels) in one patient after acute myocardial infarction (AMI) and subsequent defribriliation (A), in one patient who developed a recurrent AMI (B), and in one patient after AMI in combination with severe renal insufficiency (C). All patients received routine treatment with thrombolytic agents.*

FABP to be constant with time after AMI and of similar magnitude as the tissue ratio (from 4.1 ± 0.6 at 3 hours to 4.4 ± 0.7 at 24 hours after AMI; means ± SD for 49 patients) (12). Further confirmation of these observations was obtained in a recent study with 23 patients with AMI, for which the plasma myoglobin over FABP ratio amounted to 6.2 ± 0.4 (mean ± SD, n=23) (30).

Because the release curves of myoglobin and FABP show a similar pattern for each group of patients, our findings indicate that both myoglobin and FABP are released from

the heart and cleared from the bloodstream essentially in the same manner. In view of their low molecular masses it is most likely that myoglobin (18 kDa) as well as FABP (15 kDa) are eliminated from the circulation mainly by renal clearance (10,16). Indeed, both proteins have been found in urine from patients with AMI (13,22,23), and show elevated plasma levels during a longer period of time in case of renal insufficiency (fig 8.4C).

Aortic and cardiac surgery

Patients who underwent aortic surgery also showed a simultaneous release and clearance of myoglobin and FABP, even though peak values were recorded at a longer time period after muscle injury than in patients with AMI. We expected the former patients to have skeletal muscle injury resulting from the ischemic period during aortic surgery. Accordingly, in this patient-group the serum peak values of myoglobin and FABP generally were higher in patients with a longer period of occlusion (data not shown). In addition the serum ratio of myoglobin over FABP was 45 ± 22 (mean ± SD, n=26, 7 patients), which is within the range of ratios monitored in skeletal muscle tissue and significantly different from the ratio found in blood plasma after AMI (t-test for independent samples, AMI n=93 and aortic surgery n=26, $p < 0.001$). The slow release of proteins from skeletal muscle will relate to a lower blood flow during rest, a smaller lymph flow and a lower permeability of the endothelial barrier in skeletal muscle than in heart (2). In a study with volunteers after skeletal muscle overload, due to strenuous exercise, we also observed the ratio of myoglobin over FABP in plasma to be comparable with the ratios found in skeletal muscle tissue (25).

Patients undergoing cardiac surgery were studied as these can be expected to have both myocardial and skeletal muscle injury (24,29). In these patients the postoperative plasma curves of myoglobin and FABP were markedly different, with highest FABP concentrations found between 5 and 15 hours and highest myoglobin concentrations between 10 and 25 hours after surgery (fig 8.3B1). As a result, during this entire time interval the ratio of myoglobin over FABP first decreased from 11 (0.5 hours after surgery) to 7 (8 hours after surgery), and then increased to over 30 (24 hours after surgery) (fig 8.3B2). These data agree with earlier observations that after cardiac surgery release of enzymes from myocardium is more rapid and completed earlier in time (within 24 hours) than is release from injured skeletal muscle (> 40 hours) (29). The initial decrease of the ratio may reflect a higher relative contribution of proteins released from injured skeletal muscles (due to the operation) than from myocardial necrosis at this early point in time. Thus, assessment of postoperative changes in the ratio of myoglobin over FABP in dependence of time will give insight into the relative contribution of myocardial and skeletal muscle injury to total muscle loss.

Quantitation of muscle injury

Since in patients with AMI as well as with surgery nearly full protein release curves were recorded, it is possible to globally estimate the total amount of muscle injury, expressed as gram equivalents of healthy muscle, for each group of patients. As described elsewhere (7), the cumulative release of FABP (and of myoglobin) from muscle can be calculated using a one-compartment model, thus neglecting extravascularization of protein. Using a value of 2.6 h^{-1} for the fractional clearance rate of FABP (7) and a plasma volume of 3 litres, in the group of patients with AMI and not receiving thrombolytics the mean cumulative release of FABP is ≈ 18 mg, and in the patients receiving thrombolytics ≈ 7.5 mg, which is equivalent to ≈ 34 g and ≈ 14 g of

myocardial tissue, respectively. Similarly, in the patients who underwent aortic surgery the mean cumulative release of FABP (up to 24 h after surgery) amounts to ≈ 2.3 mg which is equivalent to ≈ 30 g skeletal muscle tissue (estimated average FABP content 0.07 mg/g). Assuming the lower part of the body to contain about 10 kg of skeletal muscle, aortic surgery is found to cause an estimated mean injury of <0.5% of the relevant muscle mass. In patients undergoing uncomplicated coronary bypass surgery, cardiac injury has been estimated to amount to about 1.5 g of myocardium compared to a loss of about 13 g of skeletal muscle (29). Since in our study patients were selected on the basis of a post-surgery increase in plasma activity of creatine kinase isoenzyme MB of more than 20 U/l, a bias was introduced and the contribution of myocardial necrosis to total muscle loss will be higher.

Clinical application and significance

The use of the ratio of myoglobin over FABP to determine the origin of protein release is illustrated by the patient who was defibrillated at arrival in the coronary care unit, an intervention resulting in a steady increase in the plasma ratio of myoglobin over FABP due to the occurrence of additional skeletal muscle injury (fig 8.4A). The latter is reflected more in the plasma curve of myoglobin than that of FABP (fig 8.4A), indicating that in this case myocardial infarct size can better be estimated from the cumulative release of FABP than of that of myoglobin.

The data from the patient who developed a recurrent infarction and the patient with AMI and renal failure show that the plasma ratio of myoglobin over FABP may help in discriminating myocardial injury alone from the situation that additional skeletal muscle injury had occurred simultaneously or shortly after AMI. After all, in the latter case similar plasma curves for myoglobin and FABP could have been observed, but the myoglobin over FABP ratio would have been quite different.

Since the plasma clearance rate of both myoglobin and FABP is rapid (7), application of the myoglobin/FABP ratio as discriminator of myocardial vs. skeletal muscle injury requires a frequent blood sampling scheme and rapid assay procedures for both proteins, which for FABP is not yet available. However, now that a rapid and sensitive monoclonal-antibody based enzyme-immunosensor assay system for FABP in plasma is being developed (17,18), the application of the myoglobin over FABP ratio can soon enter clinical practice.

Concluding remarks

The present study indicates that both myoglobin and FABP in plasma can be used as markers of loss of cardiac and/or skeletal muscle cell integrity. Both proteins show a similar pattern of release into and clearance from plasma. However, the ratio of the plasma or serum concentrations of myoglobin over FABP after myocardial injury is significantly different from that found when skeletal muscles are most likely injured, as the plasma ratio reflects the ratio in which the proteins occur in the injured tissue. Hence, measurement of myoglobin and FABP in the same blood sample and expression of their ratio is useful to determine the origin of the proteins that are released into the vascular compartment. In this way, one can discriminate between damage inflicted upon cardiac and skeletal muscle tissue.

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CHAPTER 9

GENERAL DISCUSSION

The studies described in this thesis deal with two aspects of cellular fatty acid-binding proteins in the heart. The first part of the thesis is dedicated to the role of different fatty acid-binding proteins (cytoplasmic heart-type fatty acid-binding protein, H-FABP, and the putative membrane fatty acid translocase, FAT) in the mechanism of transport of long chain fatty acids (FA) from the vascular compartment to the intracellular site of conversion. In the second part, the use of cytoplasmic H-FABP as a marker for cellular damage is studied.

MECHANISM OF CARDIAC LONG-CHAIN FATTY ACID TRANSPORT

FA are important for the heart both as energy substrates and for the formation of complex lipids (47). The low solubility of FA in aqueous environments is compensated by the presence of FA-binding proteins in most compartments of the heart. The bulk of the FA used in the heart under physiological conditions are derived from the blood plasma. These FA have to cross the endothelial barrier, the interstitial space, the sarcolemma and the cardiomyocytal cytoplasm to reach the mitochondria for metabolic conversion. In blood-plasma as well as interstitial space, albumin is present, and by binding FA this protein increases the total FA-concentration by at least four orders of magnitude (8,31,34). Several membrane-associated proteins are found to be present in the sarcolemma each of which are hypothesized to function in the transport of FA across this membrane (35,48). In the soluble cytoplasm of the cardiomyocyte, appreciable quantities are found of H-FABP, a protein which most likely is involved in the transport of FA from the sarcolemma to the mitochondrial outer membrane (14,50).

Mechanism of long-chain fatty acid transport across the endothelial cells
It was hypothesized that the transport of FA across the endothelium was also mediated by H-FABP in the endothelial cytoplasm (6,10). However, conflicting observations have been published about the H-FABP content of endothelial cells (10,26,30,32). We attemted to identify FABP types in rat heart endothelial cells, and to quantify the H-FABP and L-FABP content in this cell type. At the time of investigation, H-FABP and L-FABP were the only FABP-types known to occur in more than one tissue. Two established endothelial cell lines, RHEC50 and RHEC116, and short term cultured endothelial cells were used as a model in this study (25). In addition, immuno electron microscopy was performed on ultrathin cryosections of adult rat heart. Molecular biological studies using northern blot analysis failed to detect any H- or L-FABP mRNA, but RT-PCR showed that endothelial cells from rat heart expressed minor amounts of both H- and L-FABP. Subsequent immunochemical determination of the protein content of both H-FABP and L-FABP in isolated endothelial cells revealed that for each of the proteins its content is on the order of 1-5 ng/mg cytosolic protein. This value is more than three orders of magnitude lower than the H-FABP content in heart or the L-FABP content in liver. The very low content of H-FABP and L-FABP in endothelial cells was further confirmed by immuno electron microscopy on cryosections of rat heart using gold-labelled antibodies against H-FABP or L-FABP (chapter 3). This technique showed that the content of H-FABP in endothelial cells was at least two orders of magnitude lower than that in cardiomyocytes. Finally, an assay for FA-binding activity was developed which showed no substantial FA-binding in the cytosolic fraction of endothelial cells. Our results are in correspondence with H-FABP contents of endothelial cells reported earlier by Linssen et

al. (26), but contradict other reports on H-FABP contents in endothelial cells (10,30,32). These differences may relate to the use by the latter investigators of less specific assay methods or contaminated endothelial cell cultures.

The use of isolated endothelial cells for the molecular biological, immunological, and FA-binding studies implicate the risk of culture influences on the content of a putative FABP. These limitations are irrelevant considering the histochemical studies on cryosections of adult rat heart. However, it can not be excluded that indeed a FABP type other than H-FABP or L-FABP is present in endothelial cells in considerable amounts *in vivo* which is downregulated upon cultivation. Despite this consideration, the conclusion is drawn that rat heart endothelial cells most likely lack significant amounts of cytoplasmic FABP. This conclusion might have physiological consequences for the FA transport capacity across the endothelium.

Under physiological conditions, the heart takes up 50-100 nmol FA per gram wet weight of tissue and per min (7,9,27). To explain this FA-flux through the endothelial cytoplasm without the involvement of significant amounts of a cytoplasmic carrier such as FABP, calculations were performed to estimate the diffusional flux of FA from the luminal to the abluminal membrane. These calculations reveal that a concentration gradient of non protein bound ('free') FA across the endothelial cytoplasm of about 10 nM could result in a diffusional FA-flux of 100 nmol/min per gram of heart tissue, which is approximately the normal rate of FA-uptake in rat heart (7,9,27). Originally, we considered this FA-concentration gradient to be within the physiological range of 'free' FA concentrations in plasma and interstitial space. Therefore we concluded that FA transfer across the endothelial cytoplasm might take place predominantly by aqueous diffusion. Recent publications, however, suggest that the 'free' FA concentrations in plasma under physiological conditions are approximately 10 nM (31,34). Since the 'free' FA concentration in the interstitial space will not be zero, the FA gradient across the endothelial cell can never reach 10 nM. Assuming the 'free' FA concentration in the interstitial space to be about half of that in plasma (46), the FA concentration gradient can only be 5 nM, and the calculated diffusional flux of FA will then be on the order of 50 nmol/min per gram of heart tissue. It is important to note that these calculations are performed with several assumptions and therefore are only a rough estimation. There are some factors that can increase the calculated diffusional flux across the endothelial barrier:

1) Vesicle formation of endothelial cells. Vesicular transport is probably to slow to explain the observed FA-uptake in heart (6,47). However, this phenomenon might have other consequences for the FA transfer across the endothelium. It probably increases the surface area through which diffusion can take place and at the same time it decreases the diffusional distance, both leading to an increase in diffusional FA-flux.

2) Another point of concern in the present calculations is that they do not take into account the membrane barrier. The membrane itself has an affinity for FA and it is difficult to estimate the actual 'free' FA concentrations near the phospholipid bilayer. In addition, albumin binding proteins are reported to be present in the endothelial membrane (4,5,20,36-38), which could bind albumin-FA complexes and influence the local FA gradient. Finally, membrane-associated FA-binding proteins are shown to be present on the endothelial cell membrane and are implicated in the FA-uptake process (15,16).

Besides the factors influencing FA transfer across the endothelial cytoplasm, it is possible that still other mechanisms, like FA-transfer through the plasmalemmal bilayer as proposed by Scow et al. (39), contribute to the total FA flux through the endothelium.

Considering these various additional alternatives which can co-exist, it cannot be excluded that the main FA transport mechanism across the endothelial cell is diffusion of the unbound FA through the cytoplasm of the cell. The mechanism by which FA cross the luminal and the abluminal membrane of the endothelial cell, however, remains unclear. This lack of knowledge warrants further investigations.

Transport of long-chain fatty acids across the sarcolemma

When FA reach the cardiomyocyte complexed to albumin, they have to dissociate from the albumin carrier and have to cross the sarcolemma before they can bind to the intracellular protein H-FABP. One of the proteins hypothesized to be involved in transmembrane transport of FA is the putative fatty acid translocase (FAT) (1,17-19). When both FAT and H-FABP would be involved in the uptake and transport of FA in the cardiomyocyte, it is conceivable that the synthesis of both proteins are regulated in a similar way. A detailed study on the expression of FAT and H-FABP in rat heart and skeletal muscles revealed that these proteins showed a similar tissue distribution among heart and skeletal muscles, with highest expression levels found in heart, a somewhat lower level in soleus muscle (red, oxidative) and the lowest expression level in extensor digitorum longus muscle (white, glycolytic) (chapter 4). A similar upregulation of both proteins was found during peri- and postnatal heart development, when cardiac FA-metabolism increases (13). In addition, indications were obtained that also during streptozotocin-induced diabetes, the expression of both proteins was increased. This finding is in accordance with an earlier study by Glatz et al. (12), who found a significant increase of H-FABP protein content in rat heart during the same experimental (streptozotocin-induced) diabetes. Our results are also in agreement with a recent finding published by Greenwalt and colleagues (16), that the expression of CD36, probably a species homologue of FAT, is increased in murine models of diabetes. The co-expression of H-FABP and FAT in tissues and cell types with high FA-metabolism, and their upregulation during conditions of increased FA utilization, support their roles in FA uptake.

To find more definite proof for the role of FAT in FA-uptake across the plasmamembrane, experiments were started in which FAT was stably transfected into the cell-line H9c2 (chapter 5). This cell-line is derived from embryonic rat heart and has characteristics of both cardiac and skeletal muscle cells (21,22,28,41). H9c2 cells normally do not express FAT as was found by northern blot analysis using the FAT cDNA as probe. Fifteen different cell-lines were obtained after transfection, with different levels of FAT mRNA expression. These cell-lines offer a suitable model to study the role of FAT in FA-uptake. For instance, the hypothesis that FA-uptake is related to the expression level of FAT can now be tested.

H-FABP AS MARKER FOR CELLULAR DAMAGE

In the second part of this thesis, studies on the release of proteins from damaged cardiomyocyes are described. Detection of protein release from cardiomyocytes is generally used to determine cardiac injury (2). In addition, the amount of protein release can be used to estimate the extent of tissue injury (11). H-FABP is one of the proteins used to detect cardiac injury, e.g. following an acute myocardial infarction (AMI) (11,23,24,45). Since there was some debate in literature whether small proteins (such

as H-FABP, 15 kDa) are released earlier from damaged cardiomyocytes than are larger proteins, (i.e. CK, 80 kDa, and LDH, 140 kDa) (29,44), a study was performed to clarify this issue (chapter 7). The release of four cytoplasmic proteins, ranging in molecular mass from 15 kDa to 140 kDa, from neonatal rat cardiomyocytes was monitored under two different cell-damaging conditions, i.e. simulated ischemia and metabolic inhibition. In addition, release of one mitochondrial protein and a protein which is located partly in mitochondria and partly in the cytoplasm was measured to study the release pattern of proteins from cellular compartments other than the cytoplasm. Cytoplasmic proteins showed similar release characteristics during both simulated ischemia and metabolic inhibition. Mitochondrial or partly mitochondrial proteins showed a delayed release pattern. The latter finding is in correspondence with earlier observations made by Altona et al. (3). It is concluded that the sarcolemma does not act as a sieve through which small proteins are preferentially lost during the process of protein release. Conversely, the disruption of the sarcolemma upon cellular damage is most likely a fast process and the soluble cytoplasmic constituents all are released simultaneously.

This finding bears clinical importance in view of the search for early markers for cardiac tissue injury. Although all soluble cytoplasmic proteins under investigation were released at the same time from the damaged cells, these soluble proteins show different release characteristics in plasma samples from patients with acute myocardial infarctions (11). A delay in the protein transport from the interstitial space to the plasma compartment might cause this difference. In this process which involves lymph drainage and/or transendothelial transport, differences could exist in transport rate of small proteins, such as H-FABP, compared to larger proteins (11), see also fig 6.1.

Detection of biochemical markers in blood plasma is an important instrument which helps in differentiating patients with and without an acute myocardial infarction (AMI) (2). One of the most important characteristics of a biochemical marker for myocardial tissue injury is specificity for the heart. Both H-FABP and myoglobin are sensitive early markers for tissue injury but they do not fulfil this condition since these proteins are present not only in myocardial, but also in skeletal muscle tissue (30,43,49). We hypothesized that the ratio of the concentrations of myoglobin over H-FABP in plasma after either cardiac or skeletal muscle injury would reflect the ratio of the contents of the proteins in the affected tissue (chapter 8). Since the myoglobin/H-FABP ratio (MYO/FABP) is different in heart compared to skeletal muscles, as was expected from earlier studies (30,43,49), assessment of this ratio in plasma could differentiate myocardial from skeletal muscle injury. To test this hypothesis we measured the tissue contents of myoglobin and H-FABP in human heart and various skeletal muscles. In addition, MYO/FABP was assessed in blood samples from patients after AMI and from patients after aortic or cardiac surgery. Aortic surgery was suspected to lead to skeletal muscle injury, while cardiac surgery was shown to cause both myocardial and skeletal muscle injury (51).

The MYO/FABP ratio in heart was found to be between 4 and 6 while in skeletal muscles this ratio varies with the type of muscle and ranges from 20 to 73. After AMI, the ratio is constant during the period that the plasma concentrations of both proteins are significantly raised above there basic levels and amounts to $\approx$ 5, which is in correspondence with the ratio found in heart tissue. After aortic surgery, both myoglobin and H-FABP were released into the blood compartment. The MYO/FABP ratio in blood serum during the period that the protein levels were elevated was found to be 45 $\pm$ 22 (26 samples from 7 patients). This value is within the range of ratio's found in skeletal muscles and significantly different from the ratio found in plasma after AMI. Upon cardiac

surgery the ratio of MYO/FABP increases from ≈ 10 to over 30. These results indicate that during cardiac surgery both myocardial and skeletal muscles are injured, but that protein release from these tissues show different time characteristics. It has been described earlier that release of proteins from myocardium is faster than that from skeletal muscles (51). The fact that the plasma ratio MYO/FABP after myocardial tissue injury is significantly different from the MYO/FABP ratio found after skeletal muscle injury, makes it possible to discriminate between myocardial and skeletal muscle injury by assessment of this ratio in plasma.

FUTURE DIRECTIONS

Mechanism of cardiac long-chain fatty acid transport

Several parts of the route of FA transport from the vascular compartment to the intracellular site of conversion in the cardiomyocyte are not well understood. One of the most important issues is the control of FA uptake in the heart. Membrane proteins associated with endothelial cells and/or with the sarcolemma might play an important role in the regulation of FA permeation into cardiomyocytes. At this moment various membrane-associated proteins (such as $FABP_{PM}$, FAT and FATP) are implicated in this process of FA transfer, but their exact roles remain unclear (35,48). Transfection studies inducing high levels of expression of these proteins in cells that normally do not expres such membrane-associated proteins (e.g. H9c2 cells) might clarify some of the unsolved answers. Another manner to study the function of specific proteins in FA-uptake might be overexpressing or down regulation of these proteins in isolated cardiomyocytes. Due to the limited lifespan of isolated cardiomyocytes, stable transfections are impossible and advanced molecular biological techniques, such as adenovirus-mediated gene transfer, are necessary for efficient transfection and expression of proteins in these cells. Anti-sense techniques could be used to down regulate specific proteins and study the influence on cellular FA-uptake. Finally, the production of transgenic animals in which one of the proteins is knocked out would be an interesting model to investigate the mechanism of FA uptake.

Recently it was found that an association exists between FAT and H-FABP in the bovine mammary gland (42). It would be of high interest to study the possible interaction of these proteins in the cardiomyocyte, as this might be an important regulatory step in the FA-uptake process. Ellipsometry might provide a valuable technique to perform binding studies of H-FABP to the sarcolemma. Alternatively, binding of H-FABP to isolated FAT or the cytoplasmic part of FAT could be investigated. Co-transfection of both proteins in H9c2 cells, followed by FA-uptake studies should be compared to similar studies in which only FAT or H-FABP is transfected in this cell-line.

Antibodies raised against (parts of) the membrane-associated proteins could be useful tools to characterize the amount of specific membrane-associated proteins and their exact location in the cell. Cellular distribution of these proteins could be studied using fluorescently labelled antibodies (confocal microscopy) or gold-labelled antibodies (electronmicroscopy). In addition, these antibodies might be used as possible inhibitors in FA uptake studies, again showing the involvement of that specific protein in the FA uptake process.

Finally, it is important to realize that proteins, such as FAT and FATP might cooperate in the FA-uptake process. When studying only one of these proteins it could be difficult

to find its specific function in the complex process of FA transport from the vascular space to the intracellular site of metabolization in the cardiomyocyte.

H-FABP as marker for cardiac tissue injury

Various biochemical markers are available to detect cardiac tissue injury after an acute myocardial infarction (AMI) and to estimate the extent of this injury (2). One of the markers used to detect AMI early after onset of symptoms is H-FABP. This small protein, however, is not specific for myocardium as it is also present in appreciable amounts in skeletal muscles. We have shown that by measuring the plasma ratio of myoglobin over H-FABP, it is possible to differentiate myocardial from skeletal muscle damage. To make this finding clinically applicable it is important to develop rapid diagnostic tests to assess the myoglobin and FABP values in plasma and calculate their ratio. For this purpose, an immunosensor and a latex-based assay are being developed (33,40). The advantage of rapid detection of H-FABP and myoglobin, and subsequently calculating their ratio, warrants large clinical trials.

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SUMMARY

Oxidation of exogenous long-chain fatty acids (FA) represents the main supply of energy for the heart under physiological conditions. The FA are derived from the circulation and have to cross several barriers before they can be oxidized or, alternatively, incorporated in triacylglycerols or phospholipids. Since FA are poorly soluble in aqueous environments, several non-enzymatic FA-binding proteins (FABPs) are involved in sequestering and transporting FA throughout the body.

The present thesis deals with some aspects of the transport of FA from the circulation to the mitochondria and the role therein of membrane-associated and cytoplasmic FABPs. In addition, the use of the cytoplasmic (heart-type) FABP in the diagnosis of cardiac tissue injury was studied.

A general introduction and outline of the thesis is provided in chapter 1. A short review on the possible functions of cellular fatty acid-binding proteins in heart fatty acid metabolism is given in chapter 2. The roles of the cytoplasmic FABPs and of the more recently discovered membrane-associated FABPs are discussed. The regulation of expression of the membrane protein fatty acid translocase (FAT) and of H-FABP is reviewed in more detail, since these proteins are the main subject of the research described in this thesis.

FA transport across the endothelial cytoplasm was thought to be facilitated by an intracellular FABP. Studies described in chapter 3, however, show that the endothelial cells contain only minor amounts of H-FABP or liver-type (L-) FABP. In addition, no significant fatty acid-binding activity was found in the cytosolic protein fraction of these cells. We conclude that diffusion of non-protein bound ('free') FA is the main mechanism of FA-transport across the endothelial cytoplasm.

Several membrane-associated proteins are implicated in the transport of FA across the sarcolemma of cardiomyocytes. One of these proteins is the 88 kDa membrane protein FAT. In chapter 4, it is shown that FAT and H-FABP are co-expressed in heart and skeletal muscles, and that both proteins show a similar upregulation during heart development. Co-expression of FAT and H-FABP indicates that the proteins might have related biological functions. In addition, the expression of both proteins in tissues with high FA metabolism and the upregulation during heart development, when FA utilization increases, support the suggested roles for both proteins in FA metabolism.

To find more definite proof for the role of FAT in FA-uptake, this protein was transfected in a cell-line derived from rat heart (H9c2). This cell-line was found to have both cardiac and skeletal muscle characteristics and since H9c2 cells do not express FAT, we reasoned that this cell-line forms a suitable model system to study FAT function. Chapter 5 describes the transfection technique and shows that we achieved to stably transfect H9c2 cells with FAT, yielding several FAT expressing cell-lines. These cell-lines are now available for FA-uptake studies.

The second part of the thesis (chapter 6 through 8) handles the applicability of measuring the release of intracellular proteins to detect cellular damage. A brief introduction on cellular protein release as marker for myocardial tissue injury is given in chapter 6.

Since the issue was not settled whether small proteins are released earlier from damaged cells than are larger proteins, studies were performed on the release characteristics of proteins with different molecular masses from damaged neonatal cardiomyocytes. As is shown in chapter 7, the release of soluble cytoplasmic proteins is independent of molecular mass. It is concluded that the disruption of the sarcolemma is

a relatively fast process resulting in the simultaneous release of the soluble cytoplasmic proteins, irrespective of their molecular mass.

Chapter 8 discusses the problem that H-FABP and myoglobin are non-specific markers for heart tissue injury, since both proteins are also present in skeletal muscles. It is demonstrated, that the ratio of myoglobin over H-FABP in plasma after tissue injury reflects the ratio of both proteins in the affected tissue. Since this ratio differs between heart (5) and skeletal muscles (20-70) it can be used to differentiate myocardial injury from skeletal muscle injury.

A general discussion of the studies presented in this thesis is provided in chapter 9. The main conclusions from the research described in the present thesis are the following:

1) Since endothelial cells contain only minor amounts of a cytoplasmic FABP, the main mechanism of transport of fatty acids across the endothelial cytoplasm involves diffusion of the non-protein bound ('free') fatty acids rather than FABP-facilitated diffusion.

2) The process of fatty acid uptake across the sarcolemma is still incompletely understood and may include a role of membrane-associated fatty acid-binding proteins, like fatty acid translocase (FAT). Since fatty acids are the most important energy substrate for the heart, the uptake process is important and further research is required to gain a better understanding of the mechanism of fatty acid uptake, and its regulation, by the cardiomyocyte.

3) The release of cytoplasmic proteins from damaged cardiomyocytes is independent of their molecular mass. Proteins from other cellular compartments, however, show different release characteristics.

4) Determination of the ratio of myoglobin over H-FABP in plasma upon muscle tissue injury is a useful method to distinguish myocardial from skeletal muscle injury. Clinical trials will have to confirm our findings, and rapid tests to determine myoglobin and H-FABP values in plasma will have to be developed, before this ratio can become clinically applicable.

SAMENVATTING

Het tweede gedeelte van dit proefschrift behandelt het gebruik van de uitstort van intracellulaire eiwitten om celschade aan te tonen. Dit principe kan bijvoorbeeld gebruikt worden om de hartschade na een hartinfarct te bepalen. Hoofdstuk 6 bevat een korte inleiding over het gebruik van de uitstort van celeiwitten om hartspier beschadiging aan te tonen.

Omdat het onduidelijk was of kleine eiwitten na celschade eerder de cel uitstromen dan grotere eiwitten werd een studie verricht naar het uitstortgedrag van eiwitten met verschillende molecuulmassa's. Zoals in hoofdstuk 7 is beschreven, is aangetoond dat het uitstortgedrag onafhankelijk is van de molecuulmassa van het eiwit. De conclusie is dat de beschadiging van de celmembraan een relatief snel proces is waarna de oplosbare eiwitten in de cel tegelijkertijd worden uitgestort.

Hoofdstuk 8 behandelt het probleem dat zowel H-FABP en myoglobine niet specifieke markers zijn voor hartspierschade, omdat beide eiwitten ook in skeletspierweefsel voorkomen. In dit hoofdstuk is aangetoond dat de verhouding van myoglobin ten opzichte van H-FABP in bloedplasma na weefselbeschadiging overeenkomt met de verhouding in het beschadigde weefsel. Omdat de verhouding myoglobine/H-FABP sterk verschilt tussen hart (5) en skeletspierweefsel (20-70), kan deze goed gebruikt worden om onderscheid te maken tussen hartschade en skeletspierschade.

Hoofdstuk 9 bevat een algemene discussie gebaseerd op de resultaten van het onderzoek zoals beschreven in dit proefschrift. De belangrijkste conclusies zijn:

1) Omdat endotheelcellen slechts zeer kleine hoeveelheden cytoplasmatisch FABP bevatten is diffusie van niet aan eiwit gebonden vetzuren het belangrijkste mechanisme van vetzuurtransport door het cytoplasma van deze cel.

2) Het proces van de opname van vetzuren over de celmembraan van hartspiercellen is nog steeds niet helemaal duidelijk en waarschijnlijk spelen membraaneiwitten zoals het fatty acid translocase (FAT) hierin ook een rol. Omdat vetzuren de belangrijkste energiebron van het hart zijn, is kennis over dit opnameproces belangrijk. Vervolg-onderzoek is dan ook nodig om het mechanisme van vetzuuropname, en de regulatie hiervan, beter te kunnen begrijpen.

3) De uitstort van oplosbare cytoplasmatische eiwitten uit beschadigde hartspiercellen is onafhankelijk van hun molecuulmassa. Eiwitten uit andere cel-compartimenten vertonen echter een verschillend uitstortgedrag.

4) Bepaling van de verhouding van de concentraties van myoglobine en H-FABP in plasma na spierweefselbeschadiging is een bruikbare methode om hartspierschade te onderscheiden van skeletspierschade. Voordat deze bevinding in de praktijk toegepast kan worden zullen grote klinische onderzoeken de door ons gevonden resultaten moeten bevestigen en zullen er snelle testen ontwikkeld moeten worden om myoglobine en H-FABP in plasma te bepalen.

Het tweede gedeelte van dit proefschrift behandelt het gebruik van de uitstort van intracellulaire eiwitten om celschade aan te tonen. Dit principe kan bijvoorbeeld gebruikt worden om de hartschade na een hartinfarct te bepalen. Hoofdstuk 6 bevat een korte inleiding over het gebruik van de uitstort van celeiwitten om hartspier beschadiging aan te tonen.

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LIST OF PUBLICATIONS

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 8 juni 1965 in Ospel, gemeente Nederweert. Na het behalen van het HAVO diploma aan de Philips van Horne scholengemeenschap te Weert werd in 1982 gestart met de opleiding tot biochemisch analist aan de Zuidlimburgse Laboratoriumschool Sittard. De stage en afstudeerperiode werd doorgebracht aan de Katholieke Universiteit Nijmegen bij de werkgroep van prof. Hoenders (afdeling Biochemie), waar onderzoek werd verricht naar biologische verouderingsprocessen. In 1987 werd het diploma 'Hoger Laboratorium Onderwijs' behaald. Na het vervullen van de militaire dienstplicht (1987-1988) werd een korte periode gewerkt in het vezel-laboratorium van Rockwool Lapinus te Roermond. Hier werd met name onderzoek gedaan naar geschikte coatings van steenwolvezels voor verschillende toepassingsgebieden. In april 1989 volgde een aanstelling als biochemisch analist bij de vakgroep Fysiologie van de Rijksuniversiteit Limburg. De werkzaamheden bestonden onder andere uit de zuivering van een klein cytoplasmatisch eiwit genaamd fatty acid-binding protein (FABP), uit menselijk hartspierweefsel en het opzetten van een immunochemische bepaling om de uitstort van dit eiwit uit hartweefsel na een infarct te kunnen bestuderen. In 1992 werd hij aangesteld als AIO bij dezelfde vakgroep en deed hij onderzoek naar de rol van vetzuurbindende eiwitten in de vetzuuropname door de hartspier en het gebruik van het hart-type FABP voor de diagnose van een myocardiaal infarct. Tijdens de AIO periode zijn verschillende cursussen van de onderzoekschool Cardiovascular Research Institute Maastricht (CARIM) met goed gevolg afgelegd. In 1995 werd een werkbezoek gebracht aan de onderzoeksgroep van dr. Grimaldi aan de universiteit te Nice. Bovendien werd een subsidie van NWO verkregen voor een congresbezoek in Washington D.C. en werkbezoeken aan twee onderzoeksinstituten in New York. Vanaf april 1996 is hij als postdoctoraal onderzoeker aangesteld bij dezelfde vakgroep om in het kader van een door de Nederlandse Hartstichting gesubsidieerd project de rol van vetzuurbindende eiwitten in de vetzuuropname van het hart verder te onderzoeken.

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